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SLEEP:
EFFECTS OF CHRONIC SLEEP RESTRICTION AND ISCHEMIC STROKE
IN THE RAT

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2. Summary

The general aim of this thesis was to gain understanding in the functions of sleep by studying sleep regulation under chronic sleep loss conditions and during recovery from ischemic stroke.

Sleep is homeostatically regulated in all animal species that have been studied so far: the longer an animal stays awake, the longer and deeper it sleeps. The best characterized marker for sleep homeostasis is slow wave activity (SWA), the EEG power between 0.5 and 4 Hz in non-rapid eye movement (NREM) sleep. SWA reflects the build-up of sleep pressure: it increases in response to the duration and intensity of a prior period of spontaneous wakefulness or short term sleep deprivation (3-24h), and decreases during sleep.

However, because of an apparent lack of SWA build-up after chronic sleep restriction or deprivation, doubts have been raised about the ability of SWA to reflect chronic sleep need, and it has been suggested that sleep could be regulated by allostatic and homeostatic mechanisms under chronic sleep loss conditions.

To investigate the regulation of SWA during chronic sleep restriction (SR), we performed continuous EEG recordings during and after a 5-day SR period, in which rats were allowed to sleep during the first 4 hours of the light period, but not during the rest of the day. Most sleep was prevented during the 20-hour deprivation period, although the number of short sleep attempts increased over time. Low frequency power in the wake EEG was increased during the 20-hour deprivation period, mostly in the occipital cortex. A NREM SWA rebound was present on all SR days and during post-SR recovery. This SWA increase was most pronounced in the frontal cortex, and its magnitude was determined by the efficiency of SR. The lost SWA was compensated by the second recovery day. These results show that the homeostatic regulation of sleep is preserved under chronic sleep loss conditions, and shows much same markers as short-term sleep loss.

Although the function of sleep remains largely unclear, sleep is thought to play an important role in learning and memory formation and to be essential for at least some of the neuronal plasticity necessary for the acquisition of motor skills in the healthy brain.

Sleep disorders are common in stroke patients, and have detrimental effects on recovery of motor and cognitive functions. Moreover, disruption of sleep has detrimental effects on infarct size and expression of plasticity-related genes in rats, whereas sleep stimulants have beneficial effects in mice. Since long-term recovery after stroke depends on neuronal plasticity, strategies to improve long-term functional outcome after stroke by targeting sleep might prove to be effective.

Although alterations of sleep-wake patterns and EEG activity within vigilance states have been described in both patients and animal models, their relation to functional recovery remains unclear. We analyzed the effects of somatosensory-cortical ischemia on wakefulness, NREM and REM sleep, as well as on local motorcortical EEG activity within these states, in relation to motor function recovery in the rat. By recording EEG in an area that is not structurally damaged by the stroke, we were able to get an electrophysiological read-out of the functional remodeling in the motor cortex, without the confounding effect of mass cell death occurring in the infarcted area.

Stroke impaired motor function, but did not cause major changes in sleep and wakefulness. However, EEG power in frequencies between 7 and 25Hz was reduced in the hemisphere ipsilateral to the infarct in all vigilance states. Power in the higher frequencies of the spectrum (~13-25Hz) during sleep was related to recovery of motor function, possibly reflecting the ability of the motor cortex to display the complex activation patterns necessary to perform movements.

3. Zusammenfassung

Das allgemeine Ziel dieser Doktorarbeit war, durch Forschung von Schlafregulierung während chronischen Schlafverlusts und während der Erholung nach einem Schlaganfall, das Verständnis für die Funktionen des Schlafes zu fördern.

Schlaf ist homöostatisch reguliert bei allen Tierarten, welche bis zum heutigen Tag erforscht wurden; je länger ein Tier wach bleibt, desto länger und tiefer schläft es. Der am besten charakterisierte Indikator der Schlafhomöostase ist die langsamwellige Aktivität (slow wave activity, SWA), spektrale Leistungsdichte des Elektroenzephalograms zwischen 0.5Hz und 4Hz im NREM Schlaf. SWA reflektiert die Akkumulation des Schlafdrucks: sie steigt als Antwort auf die Dauer und Intensität einer spontanen Wachperiode oder einer kurzen Schlafdeprivation (3-24h) an und nimmt im Laufe des Schlafes wieder ab.

Jedoch stellt eine scheinbare Abwesenheit der SWA Akkumulation nach chronischer Schlafrestriktion oder -deprivation die Fähigkeit der SWA chronischen Schlafbedarf zu reflektieren zu können in Frage, und wird alternativ vorgeschlagen, dass Schlaf bei chronischem Schlafverlust durch allostatistische und homöostatische Prozesse reguliert wird.

Um die Regulierung der SWA während chronischer Schlafrestriktion (SR) zu analysieren, haben wir kontinuierliche EEG Aufzeichnungen im Laufe von und nach einer 5-tägigen SR Periode gemacht, in welcher die Ratten während der ersten 4 Stunden der Lichtperiode schlafen durften, jedoch nicht für den Rest des Tages. Der grösste Teil des Schlafs wurde in der 20-stündigen Deprivationsperiode verhindert, obwohl die Anzahl der Schlafversuche mit der Zeit zunahm. EEG Power in den niedrigen Frequenzen war erhöht während der 20-stündigen Deprivationsperiode, am meisten im Occipitalkortex. Ein NREM SWA Rebound wurde an allen SR-Tagen und im post-SR Erholungsschlaf gefunden. Die SWA Erhöhung war im Frontalkortex am stärksten ausgeprägt, und das Mass der Erhöhung wurde durch die SR-Effizienz bestimmt. Der Verlust der SWA war am zweiten Erholungstag kompensiert. Diese Resultate zeigen, dass die homöostatische Regulierung von Schlaf unter chronischen Schlafmangelbedingungen erhalten bleibt und dass sie die gleichen Indikatoren zeigt wie kurzfristiger Schlafverlust.

Obwohl die Funktion von Schlaf weitläufig unklar bleibt, spielt sie vermutlich eine wichtige Rolle beim Lernen und der Gedächtnisbildung und ist notwendig für mindestens einen Teil der neuronalen Plastizitäts, welche für den Erwerb von motorischen Fähigkeiten im gesunden Gehirn wesentlich ist.

Schlafstörungen treten häufig bei Schlaganfallpatienten auf und haben nachteilige Auswirkungen auf die Erholung der motorischen und kognitiven Funktionen. Darüber hinaus hat eine Störung des Schlafs bei Ratten nachteilige Auswirkungen auf die Infarktgrösse und die Expression von Genen die in Zusammenhang mit Plastizität stehen, während schlafstimulierende Mittel bei Mäusen vorteilhafte Wirkungen haben. Da die langfristige Erholung nach einem Schlaganfall von der neuronalen Plastizität abhängt, könnten Strategien zur Verbesserung von langfristigen Outcome nach einem Schlaganfall die auf Schlaf ausgerichtet sind, erfolgreich sein.

Obwohl Änderungen in Schlaf, Wachsein und EEG-Aktivität in diesen Zuständen sowohl bei Patienten als auch bei Tiermodellen beschrieben wurden, bleibt ihre Beziehung zur funktionellen Erholung unklar. Wir haben die Effekte einer Ischämie im somatosensorischen Kortex während Wachsein, NREM und REM Schlaf als auch auf lokale, motorische EEG Aktivität während diesen Zuständen, in Beziehung zur Erholung der motorischen Funktionen bei Ratten analysiert.

Durch die Aufzeichnung von EEG in einem Gebiet, welches keinen strukturellen Schaden durch den Schlaganfall erhalten hat, konnten wir die funktionelle Änderungen in diesem Gebiet elektrophysiologisch auslesen, ohne das Auftreten von Störeffekten von massivem Zelltod im Infarktgebiet.

Ein Schlaganfall beeinträchtigte die Motorfunktion, verursachte aber keine wesentliche Änderung im Schlaf und Wachsein. EEG Power in Frequenzen zwischen 7Hz und 25Hz wurden jedoch reduziert in der Hemisphäre ipsilateral zum Infarkt in allen Wachsamkeitsstadien. Unsere Experimente zeigten, dass Power in den höheren Frequenzen des EEG Spektrums (~15-25Hz) während des Schlafs in Verbindung mit der Erholung der motorische Funktion steht, was möglicherweise die Fähigkeit des motorischen Kortex darstellt, um die komplexen Muster welche notwendig sind zur Ausführung von Bewegungen zu aktivieren.

4. Introduction

Sleep is a state of behavioral quiescence that is characterized by reduced responsiveness to external stimuli and a quick reversibility to wakefulness, accompanied by species-specific sleep postures and timing of daily sleep periods. In addition to these behavioral criteria, it is defined by characteristic changes in the electroencephalogram (EEG) and electromyogram (EMG) in mammals and birds. (Tobler 2005)

Although all animals that have been studied so far sleep, the function of sleep remains unclear. Roles in energy conservation (Walker et al. 1980), thermoregulation (Krauchi et al. 2010), memory consolidation (Walker 2009; Diekelmann et al. 2010), neuronal plasticity (Krueger et al. 1995; Tononi et al. 2006), tissue restoration and inflammation (Krueger et al. 2001; Krueger et al. 2003) have been proposed.

Studying sleep and sleep regulation under different physiological and pathological conditions in rats, such as chronic sleep loss and recovery from ischemic stroke, can give insight into the functions of this behavior in both rats and humans and possibly lead to new treatment strategies in humans.

4.1. Vigilance states

Three vigilance states can be identified in rodents based on the EEG and EMG: wakefulness, non-rapid eye movement (NREM) sleep or slow wave sleep, and paradoxical or rapid eye movement (REM) sleep. Wakefulness is characterized by behavioral activity and cortical activation, visible as low-amplitude fast activity in the EEG combined with high and variable tonus in postural muscles. Sleep onset is associated with a slowing of the EEG and increasing EEG amplitude, the appearance of slow waves (0.5-4Hz) characteristic of NREM sleep and decreasing muscle tonus. Sleep consists of two distinct states, the slow-wave rich NREM sleep, and REM sleep, which is characterized by a low-amplitude desynchronized EEG and muscular atonia. Although REM sleep was found to have the highest arousal thresholds, making it the state with deepest sleep, cortical activation is as high as during wakefulness. A sleep period consists of alternating periods of NREM and REM sleep that are interspersed with waking periods in polyphasic animals such as rodents. The total amount of sleep varies between species, as do the relative amounts of NREM and REM sleep. For example, rats sleep ~12h in multiple naps per day, with 10-15% of REM sleep. In contrast, humans sleep ~8h in a single sleep period, with 20-25% of REM sleep.

4.1.1 Characteristics of NREM sleep

NREM sleep is characterized by the presence of three main EEG patterns: slow oscillations (~0.5-1Hz), delta waves or slow waves (1-4Hz) and sleep spindles (12-15Hz).

Sleep spindles occur during light (stage 2) NREM sleep in humans, and additionally occur shortly before the transition from NREM to REM sleep in rodents (Vyazovskiy et al. 2004a). Spindles are generated by interactions between the thalamic reticular (RE) and thalamocortical neurons (Steriade et al. 1993b; Steriade et al. 1993a; Steriade et al. 2001). Although they can be generated in the thalamus after removal of the cortex, the

corticothalamic activations are important in triggering and synchronizing spindles throughout the corticothalamic networks. Rhythmic inhibitory postsynaptic potentials generated by GABAergic reticular neurons act as pacemakers for spindle generation (Steriade et al. 1985; Steriade et al. 1987). Sleep spindles are thought to fulfill a gating function in limiting sensory input to the cortex by blocking it on the thalamic level (Steriade et al. 1969; Timofeev et al. 1996; Dang-Vu et al. 2010).

The most prominent feature of NREM sleep are the slow waves that have given this vigilance state its alternative names of slow wave sleep or delta sleep. Delta waves detected by cortical EEG electrodes have two components: a cortical and a thalamic one. Cortical delta waves reflect the synchronous firing of large groups of cortical neurons coordinated by an underlying slow oscillation (Steriade et al. 2001; Vyazovskiy et al. 2009a). The other component of delta waves is generated by thalamocortical neurons, but does not require an intact cortex (Dossi et al. 1992). The thalamic oscillation is based on two currents: a low threshold Ca^{2+} -current, and a hyperpolarization-activated cation current. Activation of the Ca^{2+} -current in the thalamocortical neuron results in an after-hyperpolarization, which activates the cation current, leading to re-triggering of the Ca^{2+} -current, resulting in a rhythmic oscillation (McCormick et al. 1990; Soltesz et al. 1991).

Slow oscillations ($<1\text{Hz}$; (Steriade et al. 1993b)) originate in the cortex, since they are present in the cortex after thalamic lesions, but absent in the thalamus of decorticated cats. These oscillations consist of the cyclic alteration of the cortical neuron between a depolarized state (up-state), when neurons show sustained firing, and a hyperpolarized state (down-state), during which they are quiet. Intracellular recordings have shown that, virtually all cortical neurons engage in the slow ($<1\text{ Hz}$) oscillation during NREM sleep (Steriade et al. 1993b; Amzica et al. 1998; Destexhe et al. 1999; Steriade et al. 2001). There is a close temporal relationship between these cellular phenomena and simultaneously recorded slow waves in the EEG (Contreras et al. 1995; Amzica et al. 1998). Specifically, the negative deflection in the surface EEG signal (or positive deflection in the local field potential, LFP) corresponds to the down-state of cortical neurons, suggesting that EEG or LFP slow waves are a reflection of near-synchronous transitions between up- and down-states in large populations of cortical neurons (Murata et al. 1963; Calvet et al. 1973; Noda et al. 1973; Burns et al. 1979; Steriade et al. 1993a; Contreras et al. 1995; Steriade et al. 2001; Molle et al. 2006; Mukovski et al. 2006; Ji et al. 2007; Luczak et al. 2007).

Sleep oscillations are not only generated by neurons, but glia cells play a role too. Glial activity does not simply reflect neuronal activity, but neurons and glia display coherent activity during slow oscillations in sleep (Amzica et al. 2002). The onset of the depolarizing phase of the slow oscillation occurred first in the neurons. In contrast, the hyperpolarized phase was initiated in glia and followed in neurons (Amzica et al. 2002). Moreover, cholinergic activation of glia led to their hyperpolarization, and was related to neuronal depolarization (Seigneur et al. 2006). The EEG oscillations during NREM sleep thus result from complex cortical interactions between neurons, as well as between neurons and glia, and from thalamocortical interplay.

4.1.2. Characteristics of REM sleep and wakefulness

REM sleep or paradoxical sleep was discovered in 1959 in cats by M. Jouvet and F. Michel. This sleep state is characterized by a complete loss of muscle tone combined with rapid eye movements and cortical activation resembling that of wakefulness (Jouvet et al. 1959).

Release of acetylcholine is increased in the cortex during REM sleep and wakefulness, resulting in the absence of long-lasting hyperpolarizations of cortical neurons during REM sleep and waking (Steriade et al. 2001). REM sleep is promoted by activation of cholinergic cells of the laterodorsal and pedunculopontine tegmental nuclei (LDTg and PPTg) in the pontine reticular formation of the brain stem, whose activation leads to forebrain activation and muscle atonia (McCarley 2004; Luppi et al. 2006; Luppi et al. 2010).

Theta activity is typical for the EEG in both REM sleep and wakefulness in animals. The theta rhythm recorded using cortical EEG electrodes in rodents stems mostly from the hippocampus. Recordings of local field potentials show that theta oscillations are largest in amplitude and most regular in frequency in the stratum lacunosum-moleculare of the CA1 region of the hippocampus, although they are also found in the CA3 region and the dentate gyrus. Oscillations in the theta frequency range have been found in cortical structures too, but none of these were able to generate theta activity when on their own.

In the simplest models of theta rhythm generation, the medial septum functions as a pacemaker that entrains the hippocampal network to express a coherent theta rhythm, leading to a single global theta oscillation (Stewart et al. 1990; Lee et al. 1994). However, it is likely that at least two (Bland 1986), and possibly more (Buzsaki 2002), independent pacemakers and many local theta-generating dipoles are involved in producing the rhythm. Indeed, several dipoles are involved in generating theta activity as measured by LFP (Montgomery et al. 2008). Moreover, each hippocampal layer generates its own theta rhythm that is somewhat independent from the global rhythm and is modulated by the animal's behavior in a layer-specific manner. Thus, although an apparently global theta rhythm can be detected by surface EEG, the origins and neuronal networks involved in generating this rhythm cannot be inferred from this, and consequently nor can the precise function of the theta oscillation during wakefulness or REM sleep.

4.2. Sleep regulation

Three processes underlie sleep regulation: a homeostatic process, as well as a circadian and an ultradian process. The ultradian process is represented by the alteration of NREM sleep and REM sleep within sleep. The timing and structure of sleep are determined by the interaction of the homeostatic and the circadian process, as described in the two-process model of sleep regulation (Borbely 1982; Achermann et al. 1993). In this model, sleep homeostasis is represented by 'process S', which rises during waking and declines during sleep. The circadian process is not dependent on prior sleep-wake history, and modulates the timing and propensity of sleep through an intrinsic pacemaker with a cycle of ~24 hours. (Day)light is the main external signal resetting the circadian pacemaker.

Sleep is homeostatically regulated in all mammalian and non-mammalian species that have been studied so far: in general, the longer an animal stays awake, the longer and deeper it sleeps. Slow wave activity (SWA, EEG power between 0.5-4Hz) in NREM sleep reflects sleep intensity, and is used as an electrophysiological marker of process S. SWA peaks at sleep onset and decreases with time spent asleep, and increases after a short (~3-24h) period of spontaneous wakefulness or sleep deprivation. Moreover, naps during the day reduce SWA in the following night. The initial increase of SWA after a period of wakefulness, followed by its monotonic decline during recovery sleep has been shown in a wide range of human and animal studies (Borbely 1982; Werth et al. 1996).

Like NREM sleep, REM sleep is homeostatically regulated, although the mechanisms for this are much less well described. There is no clear EEG marker for REM sleep intensity or pressure, making it difficult to measure and model REM sleep regulation. Benington and Heller hypothesized that REM sleep pressure increases during NREM sleep only, suggesting that the daily amount of REM sleep is determined by the amount of NREM sleep (Benington et al. 1994a; Benington et al. 1994b). However, experiments using selective REM sleep deprivation in rats showed that REM sleep propensity increases during both NREM sleep and wakefulness (Endo et al. 1997), leading to the hypothesis that REM sleep is regulated by a long term process that determines the daily amount of REM sleep, and a short-term process that determines the alternation of NREM and REM episodes within the day (Franken 2002). In contrast to NREM sleep pressure, which is manifested in increased NREM sleep intensity and duration, REM sleep pressure seems to be manifested only in increased REM sleep duration (Beersma et al. 1990; Endo et al. 1997; Endo et al. 1998a; Endo et al. 1998b).

4.2.1 Short term sleep deprivation

A period of wakefulness not only increases SWA, but also the slope of slow waves during NREM sleep. Given the relation between cortical up- and down-states and EEG slow waves, it is not surprising that the number and duration of down-states changes as a function of sleep pressure. During early sleep after spontaneous wakefulness, as well as after sleep deprivation, when large slow waves dominate the EEG, short up-states are alternated with relatively long down-states. During later sleep, up states become progressively longer (Vyazovskiy et al. 2009a).

Additionally, in early sleep, when sleep pressure is still high, most individual neurons entered and exited the up- and down-states almost synchronously with the rest of the neuronal population. When sleep pressure was lower, entry into up- and down-states was much more variable across neurons. Overall firing rates increased during a period of wakefulness and firing rates within up-states that were preceded by a wake episode are higher than in up-states preceded by a sleep episode. Both transition synchrony and the change in firing rate within a sleep episode are positively correlated to SWA. (Vyazovskiy et al. 2009a)

In addition to the effects on NREM sleep SWA described above, even short periods of sleep loss lead to pronounced changes in the wake EEG. Specifically, power in the 0.75-9Hz (delta-theta/alpha) range increases in a wake-dependent manner during sleep

deprivation in mice (Huber et al. 2000a), rats (Franken et al. 1991; Vyazovskiy et al. 2005) and humans (Cajochen et al. 1995; Aeschbach et al. 1997; Aeschbach et al. 1999; Finelli et al. 2000).

Theta power (5-8Hz) in the waking EEG may serve as another marker for process S (Finelli et al. 2000; Vyazovskiy et al. 2005). However, theta activity shows a marked circadian modulation, whereas SWA does not. The build-up of theta activity during prolonged wakefulness is related to the increase in SWA during recovery sleep, indicating that a common homeostatic process can be detected in the EEG during both sleep and wakefulness. In addition, the relative predominance of slower EEG frequencies in the wake EEG under high sleep pressure is associated with neuronal firing patterns that resemble those found in NREM sleep, possibly allowing for local relief of sleep pressure outside of NREM sleep episodes (Huber et al. 2007; Vyazovskiy et al. 2009b).

The homeostatic build-up of sleep pressure is not only affected by the duration of the preceding wake episode, but also by its quality. For example, a period of wakefulness that is dominated by exploratory behavior, which is in turn related to higher theta activity, leads to a greater SWA rebound than a period of quiet wakefulness (Vyazovskiy et al. 2005; Huber et al. 2007).

4.2.2 Chronic sleep deprivation and sleep restriction

Numerous studies have shown that SWA increases after a short period of wakefulness (see 2.1), but fewer have studied the electrophysiological consequences of total sleep deprivation periods longer than a day, or after several days of sleep restriction (SR), during which sleep is only allowed for a few hours every day. (Lancel et al. 1989; Rechtschaffen et al. 1999; Van Dongen et al. 2003; Kim et al. 2007; Akerstedt et al. 2009; Goel et al. 2009).

Four days of total sleep deprivation did not result in a SWA rebound in rats (Rechtschaffen et al. 1999). However, prolonged total sleep deprivation has major detrimental effects on the health of the rats (Rechtschaffen et al. 1999) and eventually leads to the so-called sleep deprivation syndrome, which consists of skin lesions, weight loss, excessive energy expenditure, and possibly death (Rechtschaffen et al. 1989; Rechtschaffen et al. 2002). Less severe SR protocols that allow for a small amount of daily sleep do not cause these symptoms, and enable the study of the effects of chronic sleep loss under less pathological conditions.

Restriction of sleep to 4 or 6 hours per day for 14 days had no effect on SWA during sleep opportunities in humans, although NREM SWA was increased during recovery sleep (Van Dongen et al. 2003). In contrast, in a recent study Akerstedt and colleagues (2009) (Akerstedt et al. 2009) showed that both sleep time and NREM SWA were increased during sleep periods when subjects were allowed 4 hours per day. Increased NREM SWA was also found on recovery days after the sleep restriction protocol. Repeated 12-hour sleep deprivations in rats caused increased NREM delta power in the following sleep opportunity in both the light and dark period, although this response became progressively smaller on each restriction day (Lancel et al. 1989). A more recent study showed that NREM delta power was only increased on the first day of a series of 5 days in

which rats were only allowed to sleep 4 hours per day, however, but not on subsequent days. Delta power even decreased on recovery days following the SR protocol. These conflicting results have raised doubts about the ability of SWA to reflect chronic sleep need, and suggest that sleep may be regulated by both allostatic and homeostatic processes under conditions of chronic SR (Kim et al. 2007), differentiating it from sleep regulation under normal conditions.

Enforcing chronic wakefulness in experiments, is difficult because sleep pressure increases rapidly and some sleep cannot be avoided, despite constant stimulation of the test subjects. Even in short term sleep deprivation (<24h) experiments, a small portion of sleep is maintained (usually 5-10%, refs in (Cirelli et al. 2008)), and during multiple days of total sleep deprivation rats still sleep at least 10% of the time in short microsleep episodes (Rechtschaffen et al. 2002). Importantly, slow EEG activity, including SWA and theta activity, is even present in periods of behavioral wakefulness during which the animal is moving around with its eyes open during short period of sleep deprivation (Franken et al. 1991; Vyazovskiy et al. 2005). Moreover, cortical neuronal activity changes (Vyazovskiy et al. 2009a) and brain metabolism tends to decrease (Everson et al. 1994; Wu et al. 2006) during sleep deprivation, suggesting that the brain switches into a sleep-like mode. Unfortunately the wake EEG could not be recorded continuously or detailed analysis could not be performed due to the presence of EEG artifacts in previous studies that measured SWA during and after chronic sleep deprivation or SR (Lancel et al. 1989; Rechtschaffen et al. 1999; Van Dongen et al. 2003; Kim et al. 2007; Akerstedt et al. 2009; Goel et al. 2009).

In our modern 24/7 society, chronic sleep loss is increasingly common, and has detrimental effects on cognitive function (Banks et al. 2007) and general health (Knutson et al. 2007; Meerlo et al. 2008). Thus, the issue of whether sleep remains homeostatically regulated under chronic SR is not only important for the study of sleep regulation per se, but also for understanding its negative consequences.

In the first paper in the present thesis, we present a detailed analysis of almost completely artifact-free EEG recordings during a 5-day sleep restriction period, during which we aimed to achieve the highest possible efficiency of SR.

4.3. Local aspects of sleep

Sleep is not just a global phenomenon that encompasses the entire brain, it includes in fact marked changes in specific brain areas as well.

Two species of dolphins show that sleep does not always involve the entire brain: they exhibit NREM sleep in one hemisphere only, while the other is awake. Moreover, when selectively deprived of NREM sleep in one hemisphere, the dolphins show a NREM sleep and SWA rebound in that hemisphere only (Oleksenko et al. 1992). This indicates that sleep is not only a process that is expressed by the brain and for the brain, but also that sleep pressure is increased and relieved locally. In contrast to dolphins, who rarely display NREM sleep in both hemispheres simultaneously, seals and birds are able to display both unilateral and bilateral sleep. The amount of unilateral and bilateral sleep depends whether they are currently sleeping on land or in water (seals), or on the presence of predators (birds) (Rattenborg et al. 1999; Lyamin et al. 2002; Lyamin et al. 2008). Both favor bilateral sleep when circumstances allow it.

Humans and rodents also show evidence of local regulation of sleep as well, albeit in a less spectacular way. Deep electrode recordings in humans show that while the cortical EEG may display an activation pattern consistent with sleep, subcortical regions may display wake-like patterns, and vice versa (Nobili 2010). Similar effects are seen in rats, where different cortical regions may display distinct activation patterns, particularly in the transition from wakefulness to sleep. Under conditions of low sleep pressure, this can even result in the expression of sleep-like activation patterns (i.e. up- and down-states) in single neurons surrounded by a waking brain (Vyazovskiy et al. 2009a). In humans, as well as rodents, SWA is higher in frontal than in occipital cortical regions during sleep, whereas spindle activity shows a more central predominance. Theta activity during REM sleep is most obvious in the occipital cortex in rats. The homeostatic response of SWA to sleep deprivation is also not uniform across the cortex, but shows the largest increase in the frontal cortex in humans and rodents (Oleksenko et al. 1992; Cajochen et al. 1999; Huber et al. 2000b; Marzano et al. 2009).

Regional differences in sleep and sleep regulation depend in part on morphological differences, but recent studies show a use-dependent component in sleep regulation as well. Increased stimulation of one arm by vibrations or training on a motor task during wakefulness, leading to increased activation of corresponding cortical areas, leads to higher SWA during following sleep relative to areas that had been less activated in humans (Kattler et al. 1994; Huber et al. 2004). Conversely, brain areas that have been less active, due to arm immobilization, show reduced SWA (Huber et al. 2006). Similarly, rats that are forced to use a single forepaw to retrieve food, or that have been trained to perform a motor task involving a single paw, show higher SWA in forepaw motor areas (Vyazovskiy et al. 2008c; Hanlon et al. 2009). In line with these findings related to motor activation, unilateral whisker stimulation results in higher SWA in the corresponding sensory cortex (Vyazovskiy et al. 2004b). The unilateral stimulation is accompanied by higher energy consumption/depletion, resulting in increased glucose uptake.

Given that cortical regions are differentially involved in various behaviors their homeostatic SWA rebound may vary after SR. Local effects of chronic SR are largely

unknown, although frontal and occipital areas seem to respond differentially in humans (Goel et al. 2009).

The first paper in the present thesis addresses regional differences in the response to chronic sleep restriction.

4.4. A role for sleep in learning and neuronal plasticity?

One of the current hot topics in sleep research is the role of sleep in learning and memory formation. Increasing evidence suggests that slow waves during sleep can mediate some of sleep's beneficial effects, from the prevention of cognitive impairment to memory consolidation (Huber et al. 2004; Akerstedt et al. 2009; Hanlon et al. 2009; Landsness et al. 2009). Thus, SWA may be more than just an epiphenomenon of NREM sleep and may be related to its functions.

NREM sleep slow waves may reflect local processes associated with the specific stimulation of synapses that had been insufficiently used during wakefulness to maintain neuronal connections (Krueger et al. 2003), or with general synaptic downscaling following synaptic potentiation that occurred during wakefulness, leading to an improved signal-to-noise ratio, a process called synaptic homeostasis (Tononi et al. 2006; Cirelli et al. 2008). Another proposed mechanism for the consolidation of memories during sleep is off-line replay of learned patterns: neuronal activation sequences that were learned during the day are present in a fast-forward replay during sleep (Euston et al. 2007; Sara 2010).

Support for the synaptic homeostasis hypothesis comes from experimental data showing progressively increasing firing rates during wakefulness and increased neuronal synchrony under high sleep pressure. In addition, evoked potentials obtained under high sleep pressure conditions are larger than ones obtained under low sleep pressure. Moreover, expression and phosphorylation of GluR1 subunit containing AMPA receptors is increased after a period of wakefulness and decreased after recovery sleep, indicating potentiation of synaptic transmission during wakefulness, followed by depotentiation during sleep (Cirelli et al. 2004; Vyazovskiy et al. 2008a). A net decrease in the efficacy of excitatory cortico-cortical connections can lead to decreased firing rates and synchrony, as well as a decreased number and increased duration of up and down states, resulting in decreased SWA and shallower slopes of the slow waves. The synchronous switching between up and downstates during early sleep, in combination with the neuromodulatory effect of the sleep state, may restore synaptic strength (Esser et al. 2007; Olcese et al. 2010). The same mechanisms could act on a smaller scale during wakefulness, without the need for a sustained sleep period.

Successful learning, resulting in stable memory formation, is generally thought to depend on neuronal plasticity. Repeated experiences during learning trigger a molecular cascade that induced structural changes in the activated neuronal network, including synaptic and cellular alterations, posttranslational modification of cellular proteins and modulation of gene and protein expression. Many studies have show detrimental effects of sleep loss on performance of learning tasks in humans and animal models. These studies show that at least some learning processes are sleep dependent. However, the effects and importance

of sleep for learning processes seems to depend on the type of memory task (procedural, episodic, hippocampus-dependent or independent etc.), and may affect different stages of learning differentially. Moreover, different sleep stages may have differential effects.

Long-term potentiation is one of the basic mechanisms underlying memory formation (Kandel 2001). Both REM sleep deprivation and total sleep deprivation impair LTP maintenance in rat hippocampal neurons (Romcy-Pereira et al. 2004; Kim et al. 2005). Chronic sleep disruption, which does not affect sleep time, but reduces sleep episode duration and intensity (SWA), impairs cortical and hippocampal LTP induction and maintenance, as well as performance on a spatial learning task (Morris water maze). However, sleep deprivation has been shown to facilitate LTP in the medial prefrontal cortex (Romcy-Pereira et al. 2004), suggesting that the effects of sleep loss on neuronal plasticity show regional and state dependent differences.

Gene and protein expression are differentially regulated during sleep and wakefulness in rodents (Cirelli et al. 2004; Vyazovskiy et al. 2008a) and *Drosophila* (Cirelli et al. 2005; Gilestro et al. 2009). Specifically, ~100 genes are specifically upregulated during sleep (Cirelli et al. 2004; Cirelli 2005), whereas the majority of plasticity related genes are downregulated during sleep. Both exploration-rich wakefulness, induced by exposure to an enriched environment, and training on a motor task are thought to increase neuronal plasticity and LTP. Expression of BDNF is upregulated after both of these experiences, and the amount of SWA during subsequent sleep is correlated to BDNF expression, as well as to upregulated expression of Fos and Erk (Huber et al. 2004; Huber et al. 2007; Hanlon et al. 2009). Moreover, local injection of BDNF leads to a local increase in SWA (Faraguna et al. 2008), suggesting that BDNF-mediated neuroplasticity and SWA play a role in sleep-dependent learning.

Although the majority of plasticity related genes is down-regulated during sleep, exposure to an enriched environment caused a specific up-regulation of zif-268 during REM sleep in rats (Ribeiro et al. 1999). The expression of zif-268 propagated gradually from hippocampal to extrahippocampal regions during successive REM sleep episodes, suggesting progressive re-activation of the hippocampal-neocortical network and (Ribeiro et al. 2002) consolidation of memory traces due to neuronal replay. Electrophysiological evidence also suggests reactivation of neural circuits after learning during sleep, where they display the same activation patterns as during wakefulness (Euston et al. 2007). Thus, it is possible that specific reactivation and upregulation of plasticity-related genes occurs during sleep in circuits undergoing neuronal remodeling.

4.5. Ischemic stroke and sleep

Ischemic stroke is one of the leading causes of death and disability in industrialized countries. Stroke is caused by the sudden interruption of blood flow in a focal area of the brain, resulting in rapid cell death due to lack of oxygen and energy. Currently, the only available treatment option is thrombolysis (e.g. by administration of tPA), which can rescue cells by restoring blood flow within the first hours after stroke onset. So far, neuroprotective strategies aimed at acute cell rescue have been effective in animal models, but not in patients (Auriel et al. 2010). The identification strategies aiming to improve neuronal function and functional outcome after ischemic damage, are essential for improving the life of stroke patients.

4.5.1 Sleep after stroke

Sleep disorders are common after stroke, occurring in 20-25% of patients, and have detrimental effects on neurological outcome, rehabilitation, cognitive function and quality of life (Bassetti et al. 2001; Gottselig et al. 2002; Vock et al. 2002; Bassetti 2005; Baumann et al. 2006; Siccoli et al. 2008).

Stroke patients present diverse sleep-wake disturbances, including both hypersomnia and insomnia. Experimental stroke increases NREM sleep in mice (Baumann et al. 2006), rats (Gao et al. 2010) and rabbits (Sainio et al. 1975), and reduces the amount of REM sleep (Baumann et al. 2006). SWA during NREM sleep was found to be increased after global brain ischemia in rabbits, and striatal, but not cortical ischemia in mice, and similar effects have been reported in patients (Muller et al. 2002).

Baumann and colleagues (2006) found increased theta and decreased beta activity after cortical ischemia in mice. Additionally, reduced spindle activity and interhemispheric spindle coherence has been reported in stroke patients. Recovery of spindle activity was related to better functional outcome (Gottselig et al. 2002). Thus, cortical ischemia has widespread effects on vigilance states and the neuronal activity within these states. Although some of these changes have been linked to functional outcome in patients, their role in functional recovery remains unclear.

4.5.2 Functional recovery after stroke: effects of sleep

Since functional deficits after stroke are linked to sleep-wake disturbances, and sleep is thought to be essential for at least some of the neuronal remodeling necessary for learning and motor skills (Huber et al. 2004; Hanlon et al. 2009; Walker 2009), strategies targeting sleep to improve long-term functional outcome after stroke might prove to be effective.

The damaged brain undergoes extensive neuronal remodeling after focal ischemic damage (Nudo et al. 1996; Traversa et al. 1997; Cheatwood et al. 2008; Benowitz et al. 2010). Axonal sprouting in the peri-infarct area, along with other brain areas not directly damaged by the infarct, represents an important component of post-stroke brain repair. A set of neuronal growth-associated genes has been identified to be involved in initiating, maintaining and terminating post-stroke axonal sprouting that occurs within days after

ischemic insult (Carmichael et al. 2005), that is also differentially expressed in sleep and wakefulness. Moreover, axonal sprouting after ischemia is associated with synchronous neuronal activity (Carmichael et al. 2002), one of the characteristic aspects of NREM sleep.

Only few studies have investigated causal links between sleep and ischemic brain damage or recovery of function. Post-stroke sleep disturbances, consisting of repeated 12h sleep deprivations, had detrimental effects on infarct volume and expression of plasticity related genes in rats (Gao et al. 2010). Moreover, promotion of sleep by administration of the sleep inducing compound gamma-hydroxy butyrate (GHB), improved recovery of motor function in mice, and altered expression of plasticity-related genes (Gao et al. 2008). These results suggest that the effects of sleep after stroke may in part be similar to those in the healthy brain, and including the altered expression of axonal sprouting genes.

The sudden reduction of blood flow during stroke triggers a cascade of events that includes excitotoxicity, neuroinflammation and free radical generation (Dirnagl et al. 1999). Sleep deprivation in healthy animals affects some of the same parameters: increasing levels of excitatory neurotransmitters, changing brain metabolism and increasing the production of pro-inflammatory molecules (Shearer et al. 2001; Vgontzas et al. 2004; Vyazovskiy et al. 2008a; Vyazovskiy et al. 2008b; Dash et al. 2009). However, sleep deprivation does not lead to neuronal cell death, even in animals that have died from sleep deprivation syndrome (Cirelli et al. 1999). Sleep loss may simply exacerbate the effects triggered by ischemia, and thus lead to increased brain damage.

Interestingly, both acute (1 day) and long-term total sleep deprivation (4-5 days) or selective REM sleep deprivation (6h) prior to cerebral ischemia (Hsu et al. 2003; Weil et al. 2009; Moldovan et al. 2010) protects the brain from ischemic damage, leading to less inflammation, cell death and loss of function. This suggests that prolonged wakefulness prior to ischemia may have effects similar to ischemic preconditioning by lack of oxygen, or lead to an anti-inflammatory bias in the immune response to brain damage.

4.6. Aims of the current thesis

Although sleep is a ubiquitous phenomenon, little is known about its functions. Studying sleep and sleep regulation under different physiological and pathological conditions can lead to new insights.

The first article of the current thesis focuses on the effects on chronic sleep restriction on both sleep and wakefulness, as well as on regional differences in the response to sleep loss. Recent results have raised doubt about the validity of a well established model of sleep regulation under chronic sleep loss conditions, leading to the hypothesis that sleep regulation, and perhaps sleep itself, is essentially different under conditions of acute and chronic sleep pressure. Under conditions of chronic high sleep pressure, the homeostatic regulation of sleep may break down, or be replaced by an allostatic process that results in a new setpoint for the homeostat. This casts doubt on the current assumption that sleep fulfills some fundamental need for recovery, and has an important role in maintaining proper brain function, as evidenced by both its local and global regulation. In practical

terms this might mean that, with some practice and perseverance, one could get used to sleeping very little. Thus, the issue of whether sleep remains homeostatically regulated under chronic SR is important both for the study of sleep regulation and for understanding its negative consequences.

However, due to the technical difficulties inherent to chronic SR experiments, few studies have investigated changes in EEG activity during the entire restriction period, in both sleep and wakefulness. Given the local regulation of sleep within the global behavioral vigilance states, it is possible that local sleep-like activity during behavioral wakefulness occurs under conditions of extreme sleep loss, resulting in local relief of sleep pressure and an apparent loss of homeostatic regulation. In the first paper in the present thesis, we present a detailed analysis of vigilance states and regional responses to chronic sleep loss using almost completely artifact-free EEG recordings during a 5-day sleep restriction period, during which we aimed to achieve the highest possible efficiency of SR.

Sleep is thought to have a function in regulating neuronal plasticity after learning. The recovering post-stroke brain undergoes extensive neuronal remodeling, resulting in recovery or re-learning of motor skills. Studying the effects of stroke on vigilance states and the neuronal activity therein, can therefore provide insight into the role of sleep in brain repair after ischemic damage, but also into the function of sleep in neuronal remodeling in general.

In the second paper of the current thesis, we analyzed motor function and local EEG activity during different vigilance states over the course of a 30-day recovery period after cortical ischemia. Unilateral cortical infarcts were induced in the somatosensory cortex, leaving the motor areas intact (Gharbawie et al. 2005). We compared motor cortical EEG activity and forepaw function before stroke and during a 30-day recovery period, resulting in information about the state and structural changes of the motor cortical network, as well as the functional consequences of those changes. Although no structural damage occurs in the motor cortex, its activity is severely disrupted, and extensive remodeling occurs in the recovery period (Gharbawie et al. 2005). Changes in EEG activity measured in this area are thus likely related to plasticity and recovery of motor function, with little confounding effect of the cell death and inflammation occurring in the infarct area.

5. Article 1

Sleep homeostasis in the rat is preserved during chronic sleep restriction

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Abstract

Sleep is homeostatically regulated in all animal species that have been carefully studied so far. The best characterized marker of sleep homeostasis is slow wave activity (SWA), the EEG power between 0.5 and 4 Hz during nonrapid eye movement (NREM) sleep. SWA reflects the accumulation of sleep pressure as a function of duration and/or intensity of prior wake: it increases after spontaneous wake and short-term (3–24 h) sleep deprivation and decreases during sleep. However, recent evidence suggests that during chronic sleep restriction (SR) sleep may be regulated by both allostatic and homeostatic mechanisms. Here, we performed continuous, almost completely artifact-free EEG recordings from frontal, parietal, and occipital cortex in freely moving rats ($n = 11$) during and after 5 d of SR. During SR, rats were allowed to sleep during the first 4 h of the light period ($4S^+$) but not during the following 20 h ($20S^-$). During the daily $20S^-$ most sleep was prevented, whereas the number of short (<20 s) sleep attempts increased. Low-frequency EEG power (1–6 Hz) in both sleep and wake also increased during $20S^-$, most notably in the occipital cortex. In all animals NREM SWA increased above baseline levels during the $4S^+$ periods and in post-SR recovery. The SWA increase was more pronounced in frontal cortex, and its magnitude was determined by the efficiency of SR. Analysis of cumulative slow wave energy demonstrated that the loss of SWA during SR was compensated by the end of the second recovery day. Thus, the homeostatic regulation of sleep is preserved under conditions of chronic SR.

Introduction

Sleep is homeostatically regulated in all mammalian and non-mammalian species that have been carefully studied so far: in general, the longer an animal stays awake, the longer and/or deeper it sleeps (1–4). The best characterized marker of sleep pressure in mammals and birds is slow wave activity (SWA), defined as the electroencephalogram (EEG) power between 0.5 and 4 Hz during nonrapid eye movement (NREM) sleep. SWA peaks at sleep onset and decreases with time spent asleep (3). Staying awake from ~3 to ~24 h results in progressively higher SWA levels at sleep onset, and naps during the day reduce SWA the following night (3). Slow waves reflect the synchronous firing of large groups of cortical neurons coordinated by an underlying slow oscillation, the fundamental cellular phenomenon of NREM sleep (5). Increasing evidence suggests that slow waves can mediate some of sleep's beneficial effects, from the prevention of cognitive impairment to memory consolidation (6–9). Thus, SWA may be more than just an epiphenomenon of NREM sleep and may be related to its functions.

Numerous studies have shown that SWA increases after periods of spontaneous wake or following a few hours of sleep deprivation (10–12). However, fewer experiments have measured SWA after >1 d of sleep deprivation or after several days of sleep restriction (SR), during which sleep is only allowed for a few hours every day (13–18). One study found that SWA did not increase above baseline level after 4 d of total sleep deprivation (16), and 5 d of SR resulted in a progressively smaller SWA increase in one case (15) and in no change except after the first day of SR in another (14). These results have raised doubt about the ability of SWA to reflect chronic sleep need and, more generally, have suggested that under conditions of chronic SR sleep may be regulated by both allostatic and homeostatic mechanisms (14).

Experiments to chronically enforce wake are inherently difficult, because sleep pressure increases rapidly and some sleep cannot be avoided, irrespective of stimulation. Even during experiments of short-term (<24 h) sleep deprivation some portion of baseline sleep (usually 5–10%) is always maintained (see refs. in ref. 4), and during several days of “total” sleep deprivation rats still sleep at least 10% of the time, due to “microsleep” episodes (19). Perhaps more importantly, spectral EEG analysis reveals that even during acute sleep deprivation slower activity, including SWA (0.5–4Hz) or low theta (5–7Hz) activity, leaks into periods during which the subject may be moving around with eyes open, and which are conventionally scored as wake (e.g., rats) (20, 21). Of note, when wake is prolonged beyond its physiological duration cortical neuronal activity changes (22), and brain metabolism tends to decrease, rather than increase (23, 24), suggesting that the brain may switch into a sleep-like mode. Unfortunately in previous studies that measured SWA after chronic sleep deprivation or SR (13–18) either the wake EEG could not be recorded continuously or a detailed analysis could not be performed, due to the presence of EEG artifacts.

Chronic sleep loss is increasingly frequent in our society and has detrimental effects on both cognitive function (25) and general health (26, 27). Thus, the issue of whether sleep remains homeostatically regulated under chronic SR is important not only for the study of sleep regulation per se, but also to understand its negative consequences. Here we performed continuous, almost completely artifact-free EEG recordings from frontal,

parietal, and occipital cortex in freely moving rats during and after 5 d of SR, in which sleep was allowed for only 4 h/d starting at light onset. To achieve the highest possible efficiency of SR, the automated method of sleep deprivation (disk over water) was supplemented by continuous (24 h/d \times 5 d) visual observation by the experimenter. We found that in all animals SWA showed a significant homeostatic increase on each day of SR and for at least 2 recovery d after SR. The SWA increase was more pronounced in the frontal regions, consistent with previous studies of acute sleep deprivation (12, 28, 29), and its magnitude was strongly determined by the efficiency of SR. Moreover, analysis of cumulative slow wave energy ($SWE = SWA \times \text{time}$) demonstrated that the loss of SWA during SR was compensated by the end of the second recovery day after SR. Thus, in rats, sleep homeostasis and SWA regulation appear intact under chronic SR conditions.

Results

SR Decreases Sleep Latency and Increases Sleep Consolidation.

We first tested whether during baseline all major sleep parameters, including the daily profile of SWA, did not differ in rats housed on the disk-over-water as compared with rats recorded in their home cages and found that this was the case (SI Materials and Methods and Fig. S1). Each of the 5 d of SR included 20 h of total sleep deprivation (20S⁻) followed by 4 h of sleep opportunity starting at light onset (4S⁺; Fig. S2A). During 20S⁻ the disk-over-water method, supplemented by continuous visual observation by the experimenter (Materials and Methods), was effective in preventing most, although not all, sleep in all rats (Fig. 1A). Specifically, there were almost no consolidated sleep bouts during 20S⁻, and >90% of all sleep attempts lasted <20 s (mean duration: 10.3 ± 0.1 s). NREM sleep was reduced by ~80% relative to baseline values during the same 20-h period (from 6.2 ± 0.3 to 1.1 ± 0.2 h; from 8.2 ± 0.3 to 3.2 ± 0.2 h in 24 h; Fig. S2A). In other words, only ~5% of time during 20S⁻ was spent asleep (Fig. 1B), resulting in a cumulative loss of ~25 h of

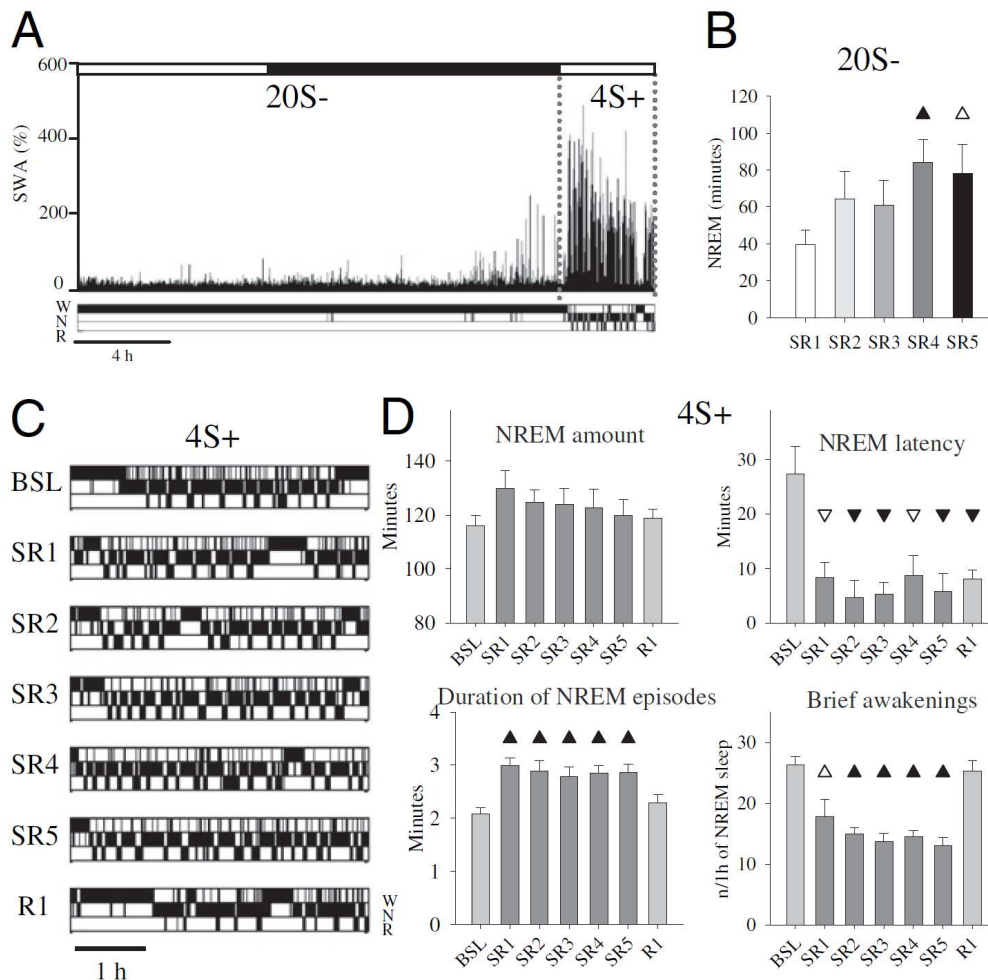


Fig. 1. (A) Hypnogram and SWA in frontal cortex (percentage of 24-h mean value in NREM sleep) during 1 of the 5 SR days in one representative rat. W, wake; N, NREM sleep; R, REM sleep. (B) Daily amount of NREM sleep during 20S⁻ (mean \pm SEM, $n = 11$): the amount of sleep increases across days [ANOVA, factor "day," $F(4,40) = 4.97$, $P = 0.002$]. Triangles indicate differences relative to the first SR day (black, $P < 0.05$; white, $P < 0.1$). (C) 4S⁺ hypnograms in a representative rat during baseline (BSL), 5 SR days, and first recovery day (R1). Note also that the sleep latency in R1 is similar to that in BSL, because rats had already slept ad libitum for 24 h before R1 (Fig. S2A). (D) NREM sleep amount, NREM sleep latency, NREM episode duration and number of brief awakenings during 4S⁺ (mean \pm SEM, $n = 11$). Triangles show differences from baseline (black, $P < 0.05$; white, $P < 0.1$, after Bonferroni correction).

sleep across the 5 SR d). REM sleep was almost completely eliminated (<0.5% of baseline, see below).

Total sleep time during 20S⁻ was higher during the last 2 d of SR relative to the first 3 d, suggesting increased sleep pressure (Fig. 1B). Changes in sleep architecture during 4S⁺ were also indicative of increased sleep propensity (Fig. 1C). Specifically, whereas the amount of NREM sleep did not change markedly, the latency to the first NREM sleep episode decreased from ~25 min to <10 min on all SR days (Fig. 1D). Moreover, sleep became more consolidated, as indicated by a ~30% increase in the duration of NREM sleep episodes and by a ~50% decrease in the number of brief awakenings (Fig. 1D). Thus, when allowed to sleep during 4S⁺, rats did so in a more consolidated way.

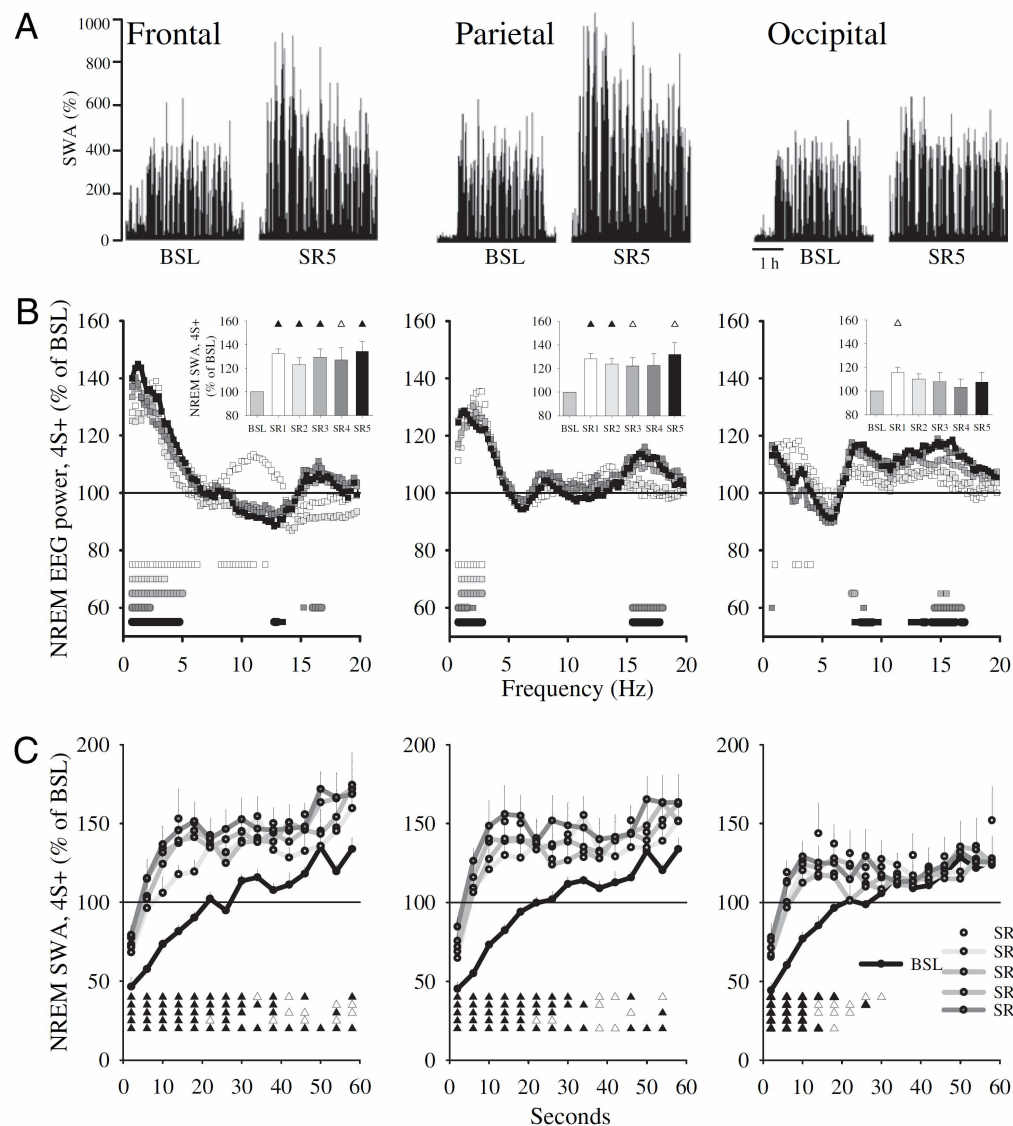


Fig. 2. (A) SWA (% of 24-h mean values in NREM sleep) during 4S⁺ in baseline and the last day of SR in one representative rat. (B) NREM sleep EEG power during 4S⁺ in the 5 d of SR plotted as percentage of the corresponding baseline period (mean, $n = 8$). Squares depict frequency bins that differed significantly from baseline ($P < 0.05$, paired t test). (Insets) Four-hour mean values of NREM SWA. Triangles show differences from baseline [paired t test; black, $P < 0.05$; white, $P < 0.1$; r ANOVA, factor "day": frontal, $F(5,35) = 6.06$, $P = 0.0004$; parietal, $F(5,35) = 5.49$, $P = 0.0008$; occipital, $F(5,35) = 1.97$, $P = 0.1$]. (C) Time course of NREM SWA during individual NREM sleep episodes in baseline and during SR (4S⁺). Triangles show differences from baseline (black, $P < 0.05$; white, $P < 0.1$; paired t test; mean \pm SEM, $n = 8$).

SR Increases NREM SWA and Does So Mostly in the Frontal Cortex.

Acute sleep deprivation is followed not only by shorter sleep latency, longer sleep episodes, and fewer brief awakenings, but also by increased NREM SWA. Thus, we asked whether SR also increases sleep pressure as measured by SWA. Indeed, on most SR days NREM SWA during $4S^+$ was significantly above baseline levels (by ~20%) in frontal and parietal cortex (Fig. 2 A and B). SWA in occipital cortex, by contrast, showed a minor increase only on the first day of SR. Changes in sleep EEG power were largely restricted to SWA in the frontal and parietal derivation, whereas in the occipital area, where changes in SWA were minor, higher frequencies (13–18 Hz) were also enhanced (Fig. 2B).

We next measured SWA changes within each NREM episode, because the intraepisode buildup of SWA is another sensitive measure of sleep pressure, becoming faster after short-term sleep deprivation in rats, mice, and humans (28, 30, 31). On all 5 SR d, the buildup of SWA during $4S^+$ was faster, suggesting a more rapid transition to the deep stages of sleep. This was the case also in the occipital cortex, despite less pronounced changes in mean SWA (Fig. 2C). Moreover, again consistent with a homeostatic process and in line with previous studies of total sleep deprivation (20), SWA was highest at the beginning of each $4S^+$ period and its decline was faster than during baseline (Fig. S3).

We also tested other sensitive markers of sleep pressure related to SWA, namely the amplitude and slope of slow waves, because they are known to increase at the beginning of the sleep period or after acute sleep deprivation (32–34). We found that during $4S^+$ both markers were higher than in baseline on all SR days and in all three cortical areas, again indicating higher sleep pressure during SR (Fig. S4 A and B). As for SWA, the differences were more pronounced in the anterior derivations.

Because we observed regional differences in the response to SR, we analyzed the origin and propagation of slow waves. As expected, we found that during baseline most (>30%) slow waves originated in the frontal cortex, whereas fewer started in more posterior areas (Fig. S4C). Interestingly, on the first day of SR the number of slow waves with frontal origin increased, while slow waves with parietal and occipital origin became less frequent, a trend then maintained across the entire duration of SR (Fig. S4D). Thus, SR increases NREM SWA and slow waves and does so more in the frontal regions.

SR Slows the Wake EEG Mostly in the Occipital Cortex.

In rodents, sustained wake also affects the wake EEG, with increases in the low-frequency range that spans SWA and low theta (5–7 Hz) activity (20, 21, 29). Such changes may reflect the buildup of sleep pressure during wake and may even partially compensate for sleep need. Thus, we next investigated how SR affects the wake EEG.

During 20S⁻ we frequently observed that the high theta (7–9Hz) activity typical of baseline wake (21) was reduced in the frontal and parietal cortex, consistent with our behavioral observation that rats on the disk do not often engage in active exploratory behavior (see below). On the other hand, EEG power in the SWA range increased mostly in the occipital derivation (Fig. 3). Thus, changes in the wake EEG during 20S⁻ seemed somehow complementary to those in NREM SWA during 4S⁺, which were largest in the frontal derivation (Fig. 2B), and mean (averaged across all rats) wake SWA for each SR day was negatively correlated with mean NREM SWA ($r = -0.81$, $P < 0.001$). In other words, the frontal cortex, which showed the highest sleep SWA during 4S⁺, had the lowest wake SWA during the preceding 20S⁻, whereas the occipital cortex showed the highest wake SWA and the lowest sleep SWA. Similar results were also observed when SWA during 20S⁻ was calculated across all three vigilance states, rather than only in wake.

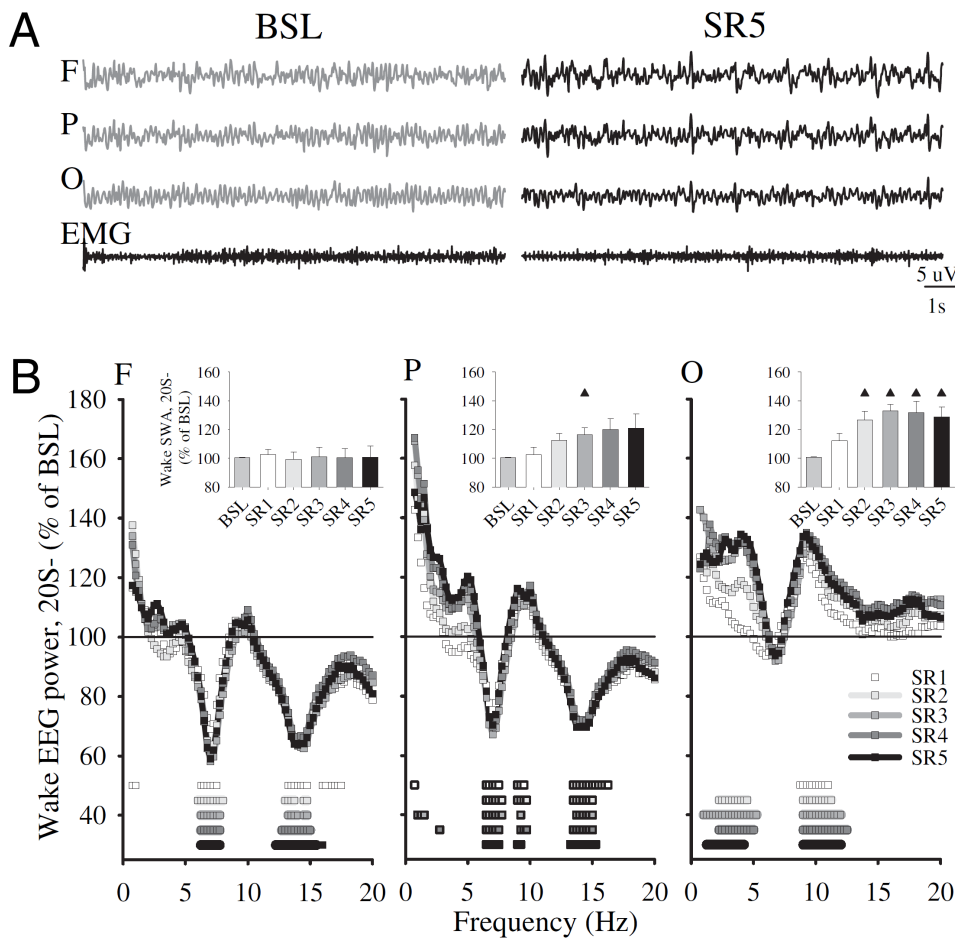


Fig. 3. (A) 12-s wake EEG traces from frontal (F), parietal (P), and occipital (O) cortex and EMG in a representative rat during BSL and day 5 of SR. (B) Wake EEG power during 20S⁻ in the 5 d of SR plotted as percentage of the corresponding 20-h baseline period (mean, $n = 8$). Squares indicate frequency bins that differed from baseline ($P < 0.05$, paired t test). (Insets) Mean 20-h values of wake SWA in baseline and SR days 1–5 ($n = 8$). Triangles show differences from baseline [paired t test, $P < 0.05$; rANOVA, factor "day": frontal, $F(5,35) = 0.49$, NS; parietal, $F(5,35) = 3.36$, $P = 0.01$; occipital, $F(5,35) = 3.79$, $P = 0.008$].

Efficiency of Sleep Loss Determines the SWA Rebound During SR.

How does the efficiency of sleep deprivation affect recovery sleep? To address this question we first investigated whether the level of NREM SWA during $4S^+$ could be predicted by the sleep/wake history during the preceding $20S^-$ episodes. Although rats were awake >90% of the time during $20S^-$, short (<20 s) sleep attempts occurred sporadically (Fig. S5 A–C). Their number was negligible during baseline (3.3 attempts/1h), increased significantly within each SR day, and was highest in the last 2 d of SR (Fig. 4A). Moreover, within each SR day, their number increased during the light period and remained high during the dark phase (Fig. S6). Thus, we subdivided all $20S^-$ phases into those with few (lowest 33%: average 6.3 attempts/1 h) and those with many (top 33%: average 22.3 attempts/1 h) sleep attempts. We found that during $4S^+$ the EEG power in the low frequencies (<9 Hz) was higher when it was preceded by a $20S^-$ phase with many sleep attempts, and lower when $20S^-$ included fewer sleep attempts (Fig. 4B). In other words, the sleepier the rats were during $20S^-$, as indicated by their repeated attempts to fall asleep, the higher was the sleep pressure during subsequent $4S^+$, as measured by NREM SWA, and the two parameters were positively correlated (Fig. 4C). Mean SWA during the sleep attempts was low ($84.35 \pm 4.8\%$ of the mean 24-h baseline value in NREM sleep), consistent with their short duration and their inability to significantly decrease sleep pressure.

Next, we asked whether the occurrence of wake SWA during $20S^-$ affects the NREM SWA during the following $4S^+$ periods. Indeed, we found that the two parameters were negatively correlated in all three cortical areas, and the effect was largely specific for the SWA range (Fig. 4D). Thus, the higher the wake SWA was during $20S^-$, the lower the sleep SWA in the following $4S^+$. To confirm this result, we subdivided the SR days into two groups of high or low SWA during $20S^-$ (calculated over all three vigilance states or NREM only) and computed the corresponding NREM spectra during the following $4S^+$ phase. We found that NREM SWA during $4S^+$ was significantly lower when preceded by a $20S^-$ phase with more slow waves ($P < 0.05$). Interestingly, we found that during each $4S^+$ phase and the 2 post-SR recovery days SWA increased also during wake and REM sleep, suggesting that the “leakage” of slow waves into other vigilance states may be another sensitive marker of increased NREM sleep pressure.

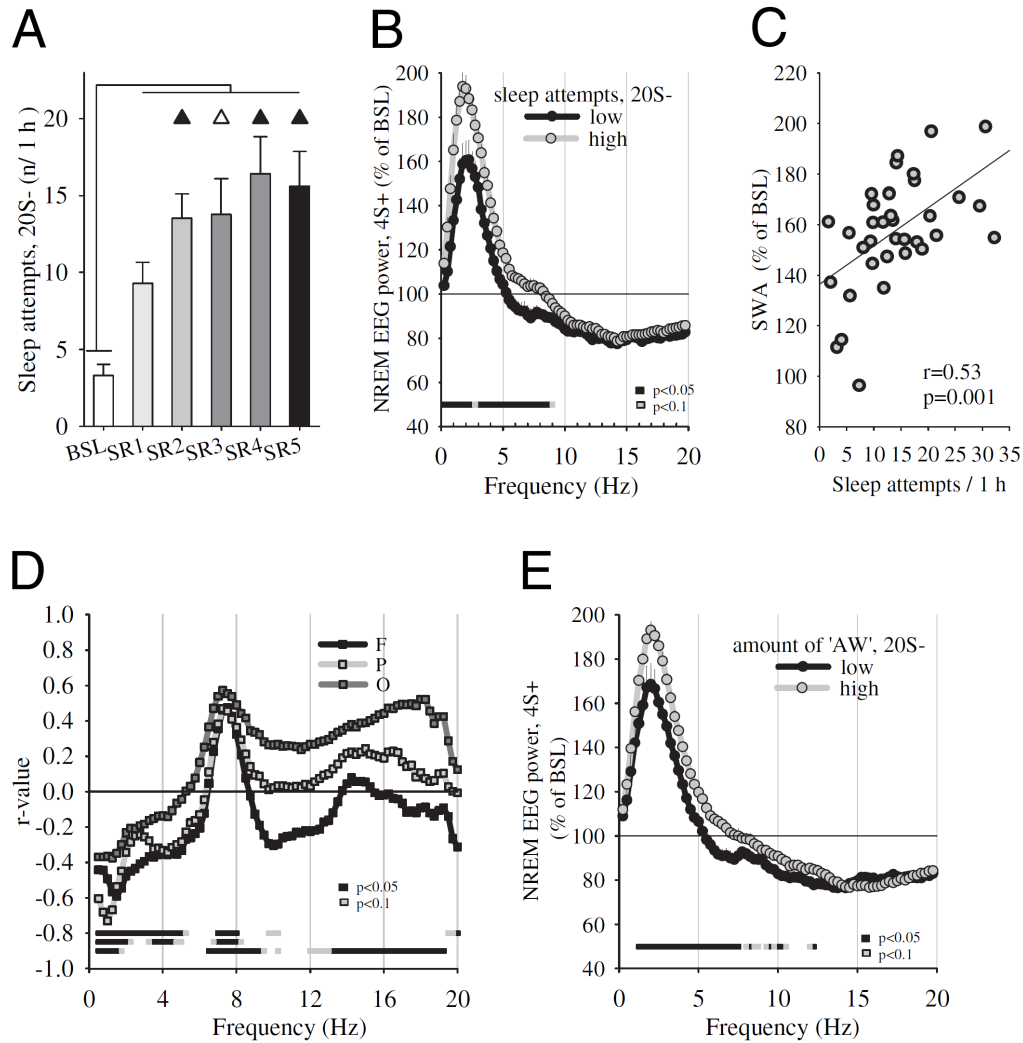


Fig. 4. (A, Left) mean number of sleep attempts (<20 s) per hour during 20S-. Triangles show differences from day 1 of SR (black, $P < 0.05$; white, $P < 0.1$). (B) NREM EEG power spectra (average of all derivations) during 4S+ plotted as percentage of baseline, shown separately for days with high (highest 33%, twelve 4S+ periods) or low (lowest 33%, twelve 4S+ periods) number of sleep attempts during 20S- (mean \pm SEM, $n=8$). (C) Relationship between the number of sleep attempts per hour during 20S- intervals and SWA in subsequent 4S+ periods ($n=8$). (D) Correlations (Pearson) between wake EEG power during 20S- and NREM SWA in subsequent 4S+. R-values are plotted for each frequency bin for F, P, and O cortex. The strong correlation in O between sleep SWA and wake beta activity may be due to the fact that the latter includes frequencies (~14–18 Hz) that are influenced significantly by the high harmonic of theta activity (~7–9 Hz), which in rats is strongest in O due to the proximity to the hippocampus. Because SWA correlates with theta activity itself, one could also expect high correlation with its harmonics. (E) NREM EEG power spectra (average of the F, P, and O) during the 4S+ periods plotted as percentage of baseline, shown separately for days with high (highest 33%) and low (lowest 33%) number of AW epochs during the preceding 20S- periods (mean \pm SEM, $n=8$).

Wake with High Theta Activity Affects NREM SWA During SR.

During SR we did not systematically score the rats' behavior, as it was highly fragmented. However, we observed that rats on the disk tried to minimize their locomotor activity by moving just enough to adjust to the slowly rotating disk and tended to be less engaged in active exploration than during baseline. Active exploratory behavior in rodents is associated with high theta activity (35), and we confirmed this fact by analyzing video recordings during baseline in a subset of animals (Fig. S5 D–F). Moreover, in all rats we compared active wake (AW) and quiet wake (QW) EEG during baseline (□5 min of recording for each state) and found that AW was reliably associated with a faster and

higher theta peak, consistent with data in mice (11) and rats (21, 36). Specifically, during baseline the EEG power between 1–6 Hz was higher in QW compared with AW, whereas the opposite was true for the EEG power between 6 and 9 Hz, which was at least 50% higher in AW (Fig. S5E). Thus, during SR we used the power ratio 6.25–9 Hz/1.25–6 Hz to distinguish AW from QW. Overall, rats spent more time in AW during SR than during baseline (Fig. S7), as expected because they were awake longer, but every day the relative amount of AW (percentage of total wake) did not differ from baseline (Fig. S7). Moreover, the average ratio 6.25–9 Hz/1.25–6 Hz within AW was actually lower during each SR day relative to baseline (Fig. S7) in frontal and parietal cortex, suggesting that when the disk rotates rats move more but may explore less.

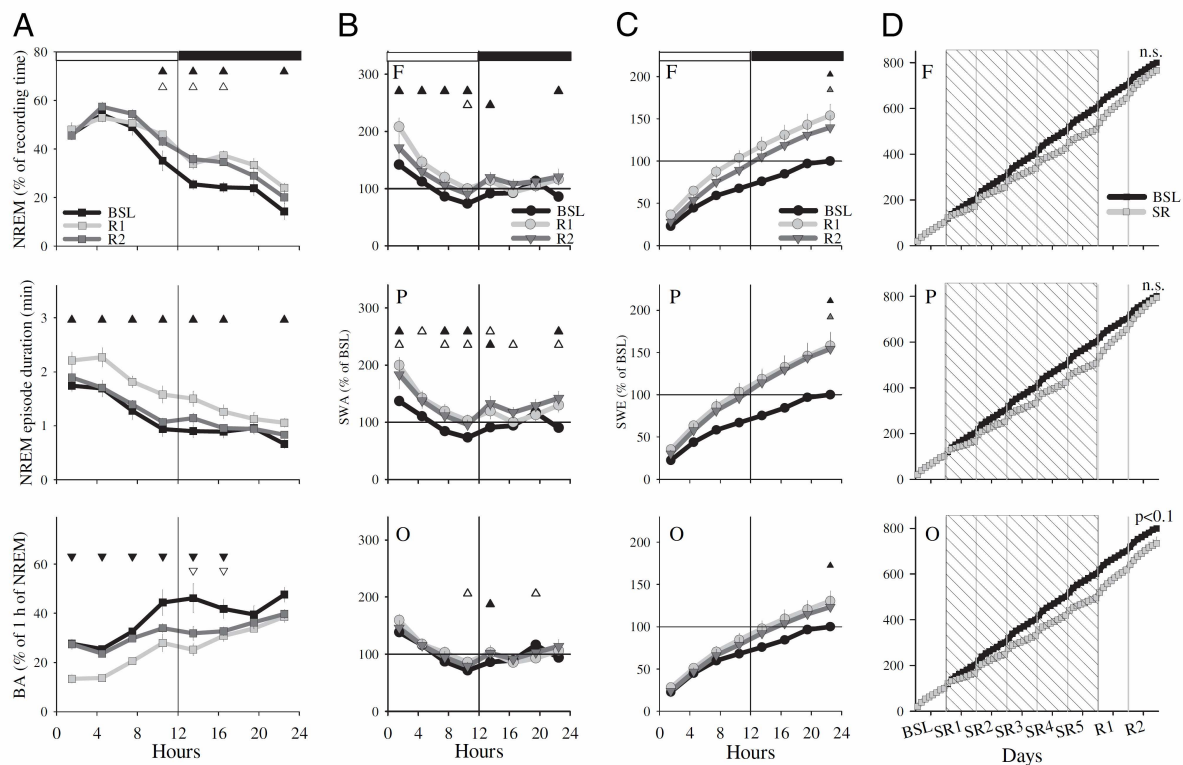


Fig. 5. (A) Time course of NREM sleep, NREM episode duration, and number of brief awakenings (BA) during BSL and 2 post-SR recovery days (mean \pm SEM, $n = 11$). Triangles show differences from baseline [Upper, BSL vs. R1; Lower, BSL vs. R2, black, $P < 0.05$; white, $P < 0.1$ after significance in r ANOVA; NREM sleep, “day”: $F(14,210) = 8.87$, $P = 9.3625e-004$, “day” \times “time interval”: $F(14,210) = 1.73$, $P = 0.05$; NREM episode duration, day: $F(14,210) = 5.87$, $P = 0.0071$, day \times time interval: $F(14,210) = 1.44$, $P = 0.13$; number of BA, day: $F(14,210) = 16.5$, $P = 1.4416e-005$, day \times time interval: $F(14,210) = 1.97$, $P = 0.0216$]. (B) Time course of SWA during BSL and 2 recovery days in F, P, and O (mean \pm SEM, $n = 8$). Triangles show differences from baseline [black, $P < 0.05$; white, $P < 0.1$ after significance in r ANOVA, interaction factors day \times time interval: frontal, $F(14,147) = 5.25$, $P = 5.3376e-008$; parietal, $F(14,147) = 3.9$, $P = 1.1684e-005$; occipital, $F(14,147) = 2.82$, $P = 9.3817e-004$]. (C) Time course of slow wave energy (SWE) in NREM sleep during baseline and 2 recovery days (mean \pm SEM, $n = 8$; symbols as in A). (D) Time course of total (cumulative) SWE computed over all three vigilance states during baseline, SR (shaded area), and 2 recovery days. Mean values ($n = 8$).

Previous studies (11, 12, 36) showed that AW leads to more intense sleep as measured by SWA. Thus, we asked whether the amount of high theta activity during $20S^-$ could affect subsequent sleep quality during $4S^+$. Indeed, the theta power between 7 and 8 Hz during $20S^-$ was positively correlated with increased sleep intensity as measured by SWA during the following $4S^+$ (Fig. 4D). Moreover, after dividing the $20S^-$ periods on the basis of the

number of AW epochs, we found that those with more AW were followed by a 4S⁺ phase with higher NREM SWA (Fig. 4E). Thus, AW, presumably dominated by exploratory behavior (35), leads to more intense sleep also during SR, consistent with the idea that homeostatic changes in sleep SWA are determined by both duration and quality of wake.

Lost SWA Is Fully Recovered After SR in Frontal and Parietal Cortex.

Signs of higher sleep pressure persisted after the end of SR, because sleep was longer and more consolidated (with longer bouts and fewer brief awakenings) throughout the first day of recovery (Fig. 5A). In frontal and parietal cortex NREM SWA was also increased, compared with baseline during the entire light phase of the first post-SR day (Fig. 5B), resulting in higher cumulative slow wave energy by the end of 24 h (SWE, SWA × time; Fig. 5C). More limited increases in NREM SWA and higher SWE were still seen during the second day after SR (Fig. 5C), suggesting that the homeostatic sleep pressure cumulated during 5 SR d took at least 2 d to dissipate.

Finally, we computed cumulative SWE throughout the entire experiment (before, during, and after SR) and compared it to the values that SWE would have reached if undisturbed sleep had been permitted during the same period. A small SWE deficit was still present by the second day after SR when only NREM SWE was included in the computation ($P < 0.1$, frontal and parietal derivation; $P < 0.01$, occipital derivation, Fig. S2C). Such deficit, however, disappeared in the frontal and parietal cortex when SWA during wake and REM sleep were also included in the computation (Fig. 5D). SWE in the occipital derivation approached baseline values but was still lower at a tendency level ($P < 0.1$). Thus, frontal and parietal cortex recovered all SWA within 2 d after SR, mostly during NREM sleep, but to some extent also during wake and REM sleep.

Changes in REM Sleep During and After SR.

REM sleep was almost completely suppressed during 20S⁻ and showed a large rebound on each of the five 4S⁺ periods, when REM sleep latency decreased and number and duration of REM sleep episodes increased (Fig. S8A). REM sleep EEG spectra during the five 4S⁺ periods showed a large increase in power in most frequencies including SWA, especially in frontal and parietal cortex, whereas EEG power in the alpha/theta range (6.25–10 Hz) increased less or not at all (occipital cortex; Fig. S8B). In previous studies (37, 38) an increased ratio SWA to alpha/theta during REM sleep was suggested to reflect the buildup of REM pressure, but during our SR experiment this ratio either did not change (frontal and occipital cortex) or increased, but not progressively (parietal cortex). Large increases in REM sleep were also present after SR and as a result, almost all REM sleep lost during SR was recovered by the end of the second post-SR day (Fig. S8C).

Discussion

We show here that all of the established markers of sleep pressure that we measured responded to chronic SR as they do after acute sleep loss, namely with an increase in sleep attempts, a decrease in sleep latency and brief awakenings, and an increase in the duration of sleep episode, SWA, and the amplitude and slope of NREM slow waves. It is worth pointing out that constant visual observation of the rats for 5 consecutive days was required to prevent sleep as successfully as possible. This may explain why an SWA rebound (and other measures of homeostatic sleep pressure) was not observed in some previous studies that used similar SR regimes, but in which these somewhat “extreme” measures were not taken. Indeed, in the most recent study (14) the overall average SWA during the 20S⁻ phases was not significantly lower than during the same time period during baseline, when rats could sleep ad libitum, and in a previous total sleep deprivation study SWA during 4 d of presumed “total waking” was 65.5% of the total 24-h SWA during baseline, when rats slept at least for 12–14 h (16). In contrast, a recent human study (13) where subjects were allowed to spend time outdoors (which may have helped them to stay awake more effectively), showed a sleep rebound on each of the 5 d of SR. Another difference between our study and that of Kim et al. (14), who used the same SR protocol, is that we analyzed the wake EEG for the entire duration of the experiment. This is crucial to interpret changes in SWA, because the latter is exquisitely sensitive to the sleep/waking history of the previous several hours (e.g., ref. 12), and it declines quickly during sleep (39).

Do the observed changes in SWA suggest that the homeostatic mechanisms of sleep regulation remain intact during SR? One could argue that if so, the increasing sleep pressure should have resulted in progressively larger SWA rebounds during the five 4S⁺ phases. However, this should not necessarily be the case. Already after the first SR day rats were asleep ~3 of the 4 allowed hours, with almost a third of total sleep consisting of REM sleep, and spent the remaining time mostly eating and grooming in preparation for sleep. In our experience, even rats sleep deprived for 1–2 wk will not forgo these activities in the first 1–2 h of recovery. Moreover, both the amplitude and the incidence of slow waves, and thus SWA, cannot increase indefinitely, as they are limited by the extent of the recruitment of local neural populations in the slow oscillation (33, 34). On the other hand, if SWA during SR is homeostatically regulated its levels during each sleep opportunity window should be determined by the sleep pressure accumulated during the preceding wake interval. We found that this was the case: the more effective we were in enforcing wake during each 20S⁻, the larger was the sleep SWA rebound during the following 4S⁺. More stringently, SWE analysis demonstrated that all SWA lost during SR was recovered within the first 2 d of recovery. Importantly, although most (~90%) of the recovery occurred during NREM sleep, some also took place in REM sleep and wake, indicating that sleep pressure during SR is high enough to force the leakage of slow waves into other behavioral states. Thus, it appears that homeostatic mechanisms of sleep regulation as measured by SWA remain intact during and after 5 d of SR, although we cannot rule out that longer periods of sleep loss would have produced different results. A period of 5 d was chosen to follow as closely as possible the design used by Kim and

colleagues (14), and because it mimics what often happens in real life, where sleep is restricted during the week days and recovered during the weekend.

We found that the SWA rebound after chronic SR was largest in the frontal cortex and lowest in the occipital cortex, consistent with the results of a recent study of chronic SR in humans (18) and of several studies of total sleep deprivation (29, 40, 41). The frontal predominance in SWA is also observed in baseline conditions (29, 40–42) and may result from stronger cortico–cortical connections in the anterior as compared with posterior cortex (41), leading to stronger neuronal synchronization and a preferential origin of sleep slow waves in frontal areas (43, 44). Recent evidence suggests that SWA reflects the extent of neuronal activity and plasticity that occurred during the prior wake period (45–48). Thus, higher frontal SWA may also reflect heavier neuronal use/plasticity in the anterior as compared with posterior cortex, although this remains to be demonstrated. In summary, several not mutually exclusive mechanisms may underlie the frontal predominance of SWA, but their respective role remains to be tested experimentally.

Our results show that the intrusion of slow waves during wake slows down the build up of sleep pressure and does so in a region- specific manner, a finding never reported before, to our knowledge. From a practical point of view this means that, especially in experiments in which wake is prolonged for many hours or days, the analysis of the wake EEG in different cortical areas is important to understand the sleep homeostatic response. From a functional point of view, an intriguing possibility is that “wake slow waves” may decrease sleep need because they provide, at the cellular level, at least some of the benefits of sleep slow waves. Although highly speculative at this time, this hypothesis can be tested experimentally, for instance by measuring whether molecular and electrophysiological markers of synaptic strength, which decrease during sleep (49), are also decreased during wake with sustained increase in SWA. It is worth remembering, however, that an increase in wake SWA, whether caused by prolonged wake or by certain drugs (e.g., anticholinergic agents) (50), consistently results in cognitive deficits (4).

Materials and Methods

Animals.

Male WHY rats ($n = 11$) were implanted for EEG recordings as described (36). Animal procedures followed National Institutes of Health guidelines and facilities were approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison. SR (20S⁻) was enforced using the disk-over-water method (16) (SI Materials and Methods).

EEG Scoring.

Behavioral states were scored as described (36) (Fig. S5A) using three EEG signals (frontal, F; parietal, P; occipital, O) and muscle (EMG) signal. Because one of the main goals of the study was to assess the effects of SR on the wake EEG, we took extreme care in excluding from the latter even very short episodes (<2 s) containing slow waves, and epochs were scored as NREM sleep if the EEG amplitude was >2 -fold higher than wake (Fig. S5B). Sleep attempts were defined as sleep episodes <20 s preceded and followed by wake episodes >20 s (Fig. S5C). Brief awakenings were defined as short episodes of arousal <16 s (20). Sleep latency was defined as the time elapsed between the onset of the 4S⁺ period and the first NREM sleep episode >16 s. Epochs with movement or technical artifacts were excluded from spectral analysis (baseline: $4.6 \pm 1.2\%$, of which $95.6 \pm 1.3\%$ occurred in wake; SR: $5.9 \pm 1.6\%$, $96.7 \pm 1.5\%$ in wake). Spectral analysis was performed on a subset of rats ($n = 8$), in which EEG signals remained stable throughout the entire 8-d experimental period.

Signal Processing and Statistical Analysis.

Data acquisition and analysis was performed as described (34, 36) using MATLAB (Math Works). Detection of individual slow waves was performed according to ref. 34 and SI Materials and Methods). Changes in sleep variables, EEG power in selected frequency bands, or EEG spectra were tested with one-way ANOVA for repeated measures (rANOVA) with factor “day,” or with two-way rANOVA with factors day and “time interval.” Contrasts were tested by two-tailed paired t tests for those cases where rANOVA was significant. The relationships between the efficiency of 20S⁻ and subsequent sleep during 4S⁺ were assessed with linear correlation analysis.

Acknowledgements

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Supporting Information

SI Materials and Methods

Animals and Surgical Procedures. Male Wistar Kyoto rats (3–4 mo, 250–350 g; Charles River) were maintained on a 12:12 light–dark cycle (lights on at 10:00 AM; room temperature 23 ± 1 °C), with food and water available ad libitum for the duration of the experiment. For electroencephalographic (EEG) recordings rats were implanted bilaterally with epidural screw electrodes over the frontal (anteroposterior, AP, +2.5 mm from bregma; mediolateral, ML, 3 mm), parietal (AP –2.5 mm; ML 5 mm), and occipital (AP –7 mm; ML 3.5 mm) cortex. Ground and reference electrodes were implanted over the cerebellum. Two stainless steel wires inserted into the neck muscles served as electromyogram (EMG) electrodes. After surgery, rats were allowed to recover in individual Plexiglas cages (36.5 × 25 × 46 cm) for at least 2 d before they were transferred to the disk (see below). For the final EEG analysis the “best” electrode (most stable signal throughout the experiment, lowest number of artifacts) for each cortical area was selected in each rat (left hemisphere: frontal, $n = 6$, parietal, $n = 5$, occipital, $n = 6$; right hemisphere: frontal, $n = 5$, parietal, $n = 6$, occipital, $n = 5$). All animal procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and facilities were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison.

Sleep Restriction (SR). Twenty hours of SR (20S[–]) were enforced using the disk-over-water (DOW) method, a well-established technique for long-term sleep deprivation (1). The rat is placed on one-half of a rotating platform (18 in in diameter) located above a tray filled with water. When the DOW rotates the rat must keep moving to avoid falling into the water. After recovery from surgery rats were transferred to the DOW, which remained stationary for several days, then was continuously rotated for 15 min immediately after light onset for 3–4 d to habituate the animals to the movement. The experiments began only after all rats were well adapted to this procedure and their sleep–wake cycle had fully normalized (see below, baseline sleep on the DOW; Fig. S1). A 24-h baseline was then recorded, followed by 5 d of SR. During SR the DOW was stationary for the first 4 h after light onset, allowing undisturbed sleep (4S⁺) and rotated during 20S[–], preventing consolidated periods of sleep. SR was followed by 2 d of undisturbed recovery on the stationary DOW. We tested different protocols of DOW rotation (0.6 or 2 rpm with random change in direction every 20 s; 2.5 rpm with no change in direction) and found that their effectiveness in preventing sleep was similar, but suboptimal. Indeed, we soon realized from online EEG observation that sleep could be almost completely prevented only if there was constant monitoring by the experimenter during 20S[–]. This was needed to quickly repair the DOW if it stopped working, and, more importantly, to anticipate any attempt by the rat to go to sleep even for just a few seconds by introducing novel objects. Moreover, we found that several rats, if left alone, adopted strategies that allowed them to sleep on the rotating DOW without falling off. In these cases, objects were hung from the cage walls to distract the animal or physically impede their strategy. During repairs, rats were placed in cages with clean bedding, food, and novel objects. EEG and EMG were still continuously recorded and monitored during these periods. To assure that the animals had sufficient time for eating, drinking, and grooming the DOW was stopped

every few hours for 10–15 min under the supervision of the experimenter. The DOW was also stopped for cleaning for 10–15 min before 10 AM, to allow undisturbed recovery immediately at light onset. Daily monitoring of the animals' physical appearance did not find obvious signs of discomfort or debilitation. A small weight loss (~10% by the end of the experiment) occurred in a few animals.

Scoring of Vigilance States. During baseline, NREM sleep, REM sleep, and wake were scored offline by visual inspection of 4-s epochs (SleepSign; Kissei Comtec) according to standard criteria (Fig. S5A). Three EEG signals (frontal, parietal, and occipital) and the EMG were used for scoring. Wake was characterized by low voltage, high frequency EEG pattern and phasic EMG activity. NREM sleep was characterized by the occurrence of high amplitude slow waves, spindles, and low tonic EMG activity. The EEG during REM sleep resembled that of wake, but only heartbeats and occasional twitches were present in the EMG signal. Since one of the main goals of the study was to assess the effects of SR on the wake EEG, we took extreme care in excluding from the latter even very short episodes containing slow waves. Thus, although according to standard scoring rules a 4-s epoch containing <2 s of slow waves would still be counted as “wake,” we used a more conservative approach. Specifically, epochs were scored as NREM sleep when the amplitude of the EEG was at any point during the 4 s increased more than 2-fold compared with the typical levels observed during wake EEG. This occurred due to the sporadic (1–1.5 s) occurrence of slow waves similar to those typical of NREM sleep (e.g., Fig. S5B). Isolated slow waves (<2 s) were also sometimes present in REM sleep during $4S^+$ and recovery, but such cases were not considered an interruption of REM sleep unless a clear-cut transition to NREM sleep was apparent, i.e., the slow waves comprised more than half of the epoch and were present also in the following epochs. Behavioral observation revealed that epochs with increased SWA during $20S^-$ were often associated with immobility and an attempt to assume a sleep-like posture. Such episodes were considered sleep attempts if they were <20 s and were preceded and followed by wake episodes >20 s (Fig. S5C). NREM sleep episodes instead were defined as periods of NREM sleep lasting >16 s, preceded or followed by wake or REM sleep (2-4). Brief awakenings (5)(Franken et al. 1991) were defined as short episodes of arousal, characterized by flattening of the EEG signal in all three derivations and concomitant increase of the EMG tone lasting ≤ 16 s and preceded and followed by NREM sleep. Sleep latency was defined as the time elapsed between the beginning of the $4S^+$ period and the first NREM sleep episode lasting >16 s. During offline scoring the epochs with movement or technical artifacts were carefully marked to be excluded from spectral analysis. The absence of artifacts was verified post hoc on each individual 24-h profile of SWA in each derivation. During baseline, only $4.6 \pm 1.2\%$ of all epochs were scored as artifacts ($95.6 \pm 1.3\%$ of them occurred during wake), and during SR the number was only marginally higher ($5.9 \pm 1.6\%$, $96.7 \pm 1.5\%$ in wake). Three rats in which one or more EEG channels showed deterioration during the experiment (overall decrease in total EEG power and signal amplitude >20%) were excluded from spectral analysis but included in the analysis of vigilance states, since the latter could still be reliably determined.

Signal Processing and Analysis. Data acquisition was performed with the Multichannel Neurophysiology Recording System (Tucker-Davis Technologies, TDT). EEG and EMG were collected continuously at a sampling rate of 256 Hz (filtered between 0.1–100 Hz). For

staging, signals were loaded in custom-made Matlab programs using standard TDT routines, and subsequently transformed into the European Data Format (EDF) with Neurotraces software (www.neurotraces.com). Video recordings were performed continuously with infrared cameras and stored in real time. EEG power spectra were computed by a fast Fourier transform (FFT) routine for 4-s epochs (SleepSign, Kissei, 0.25-Hz resolution). Detection of individual slow waves was performed on the EEG signal after band pass filtering (0.5–4 Hz, stopband edge frequencies 0.1–10 Hz) with MATLAB `filtfilt` function exploiting a Chebyshev type II filter design (MATLAB, Math Works). Filter settings were optimized visually to obtain the maximal resolution of wave shape, as well as the least intrusion of fast (e.g., spindle) activities (6-8). Slow waves were detected as negative deflections of the EEG signal between two consecutive positive deflections below the zero crossing separated by at least 0.1 s. The peak-to-peak amplitude of the first and second segment of the wave and the slopes (mean first derivative of the first and second segment) were computed for each individual wave during NREM sleep. To investigate spontaneous traveling waves, large slow waves (>median amplitude) were detected in each of the three derivations (either frontal, parietal, or occipital = “origin” derivation). The slow wave negative peak in the origin was used as reference, and the slow wave was considered to be propagating if (1) a negative peak in the other two derivations occurred within the following 50–200 ms and (2) the corresponding waves were at least 1/5 of the amplitude of the origin wave (7).

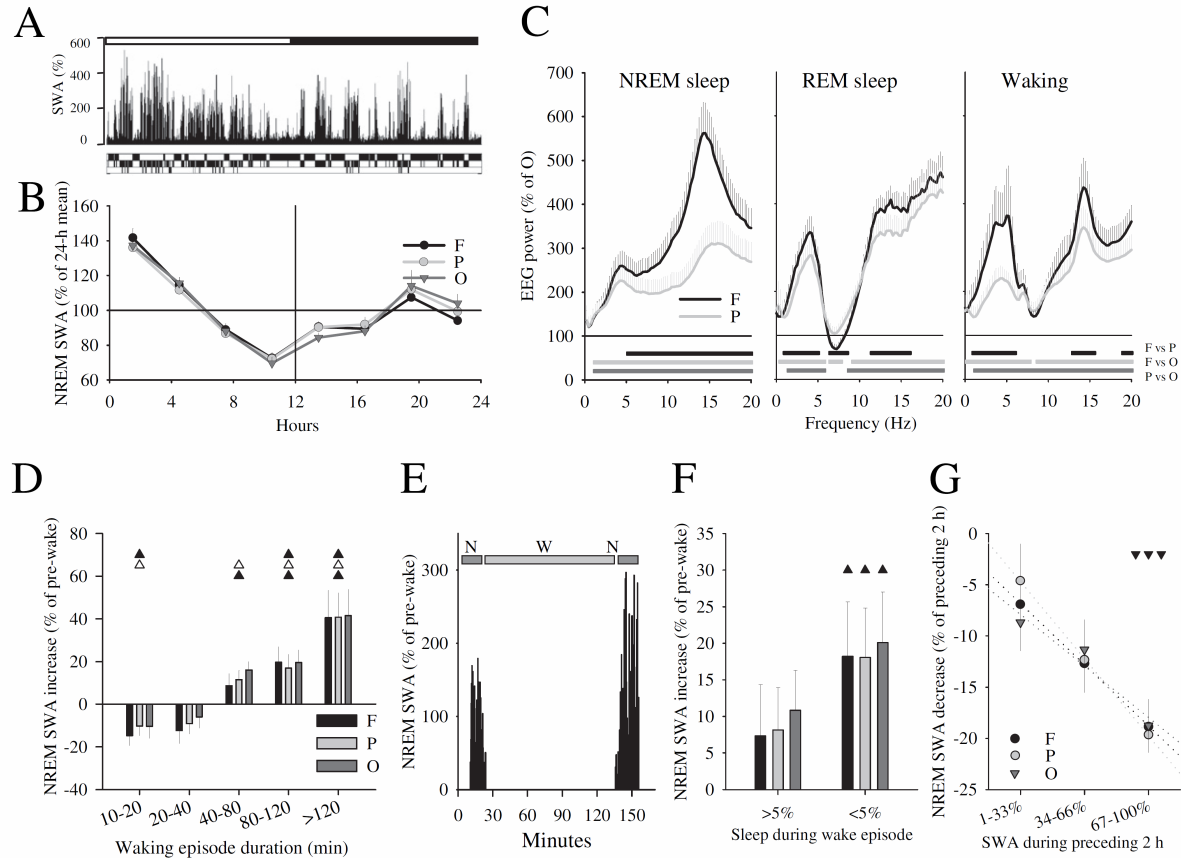


Fig. S1 (A) SWA (percentage of 24-h mean value in NREM sleep) and hypnogram of the 24-h baseline in one individual rat. Note high values of SWA at the beginning of the light period. (B) Time course of NREM SWA in the frontal (F), parietal (P), and occipital (O) cortex during baseline. Mean values \pm SEM, $n = 11$. (C) Baseline EEG power spectra in the frontal and parietal derivation expressed as percentage of the EEG spectrum in the occipital derivation. Mean values \pm SEM, $n = 8$. Horizontal lines below the curves depict frequency bins where EEG power differed significantly across derivations ($P < 0.05$, paired t test). (D) Effects of spontaneous wake episode duration (>10 min) on NREM SWA. Bars show the change of SWA (30-min intervals consisting of at least 33% of NREM sleep before and after wake episodes) from prewake levels. Mean values \pm SEM, $n = 11$. Triangles depict significant changes ($P < 0.05$, paired t test). (E) Typical example of the increase in NREM SWA after a long spontaneous wake episode. (F) Change in NREM SWA in the frontal derivation after spontaneous wake episodes (mean values \pm SEM, $n = 11$ rats). All wake episodes were subdivided into those with short sleep intrusions ($>5\%$ sleep) and those containing $<5\%$ of NREM sleep. Triangles depict significant differences from prewake levels (G). Decrease of NREM SWA from one 2-h interval to the next as a function of the level of SWA during the first interval (mean values \pm SEM, $n = 8$).

Baseline Sleep on the DOW.

Visual observations and quantitative analysis revealed that all rats were well adapted to the DOW before the experiment began, with no abnormalities in their behavior and sleep/wake cycle (mean values in hours \pm SD: light period, wake: 4.86 ± 1.24 , NREM sleep: 5.53 ± 0.89 , REM sleep: 1.21 ± 0.42 ; dark period, wake: 8.43 ± 0.86 , NREM sleep: 2.63 ± 0.42 h, REM sleep: 0.38 ± 0.14 ; light vs. dark, $P < 0.01$ for all three vigilance states). Measures of sleep architecture (e.g., duration and frequency of sleep episodes, number and distribution of brief awakenings, see Figs. 1D and 5A), and NREM SWA profile (Fig. S1 A and B) were also similar to those reported in rats recorded in their home cages (5, 9). The frontal, parietal, and occipital derivations showed previously reported regional EEG differences during wake and sleep (9-10): total EEG power was higher in the frontal and parietal derivations as compared with the occipital derivation, with the exception of the theta frequency band in wake and REM sleep (Fig. S1C). In NREM sleep EEG power in

SWA (0.5–4.0 Hz) and spindle range (10–15 Hz) was greater in frontal than in posterior regions (Fig. S1C). The homeostatic changes of NREM SWA also exhibited the previously described frontal predominance (10–11) frontal vs. parietal, 141.7 ± 5.4 vs. 136.0 ± 4.3 , % of mean 24-h BSL, $P < 0.01$). To investigate whether the changes of SWA during baseline were homeostatic, e.g., directly related to the sleep/wake history, we first computed the amount of SWA after spontaneously occurring wake episodes >10 min. As previously reported in mice (12), longer wake episodes were consistently followed by more intense and consolidated sleep, irrespective of time of day (Fig. S1 D and E). Moreover, wake episodes with only few sleep attempts were followed by significantly increased NREM SWA, whereas SWA did not increase after wake episodes containing $>5\%$ of NREM sleep (Fig. S1F). Finally, we found that intense sleep, characterized by high values of SWA, was more effective in dissipating sleep pressure: thus, the extent of the SWA decrease from one 2-h interval to the next was predicted by the value of SWA during the first interval (Fig. S1G).

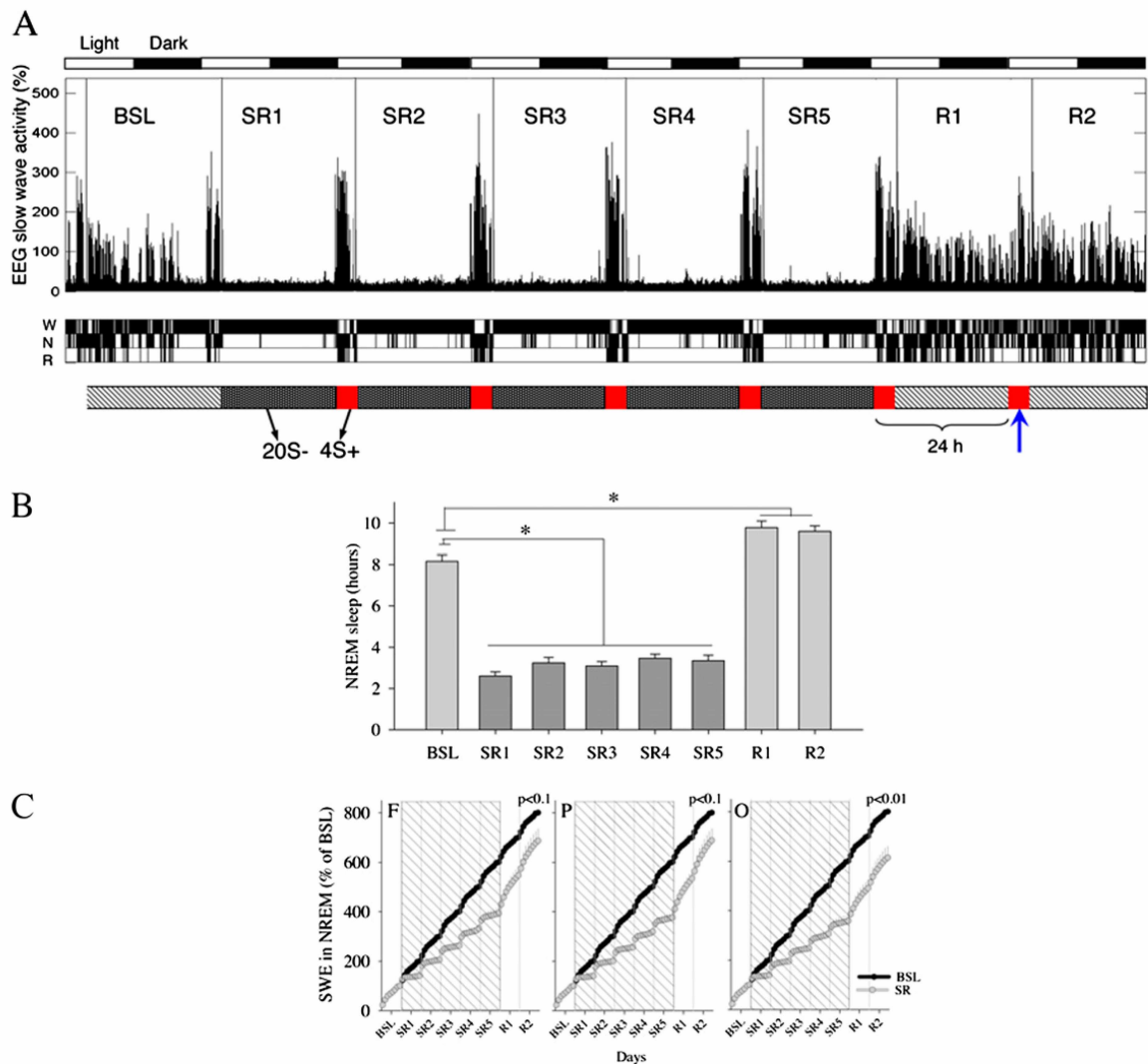


Fig. S2 (A) Schematic of the experimental design: the SR experiment starts with the 20S⁻ period, 4 h after lights on, followed by the 4S⁺ (red block), which therefore occurs during the following light period. Note that the R1 red block (blue arrow) occurs after rats had already slept ad libitum for 24 h. (B) Amount of NREM sleep during 24 h (mean \pm SEM, $n = 11$). Asterisks indicate differences relative to the first SR day [$P < 0.05$, paired t test after significance in rANOVA, factor “day”: $F(7,70) = 202.3$; $P < 0.0001$]. (C) Time course of total (cumulative) SWE in NREM sleep during baseline, SR (shaded area) and 2 recovery days. Mean values, $n = 8$.

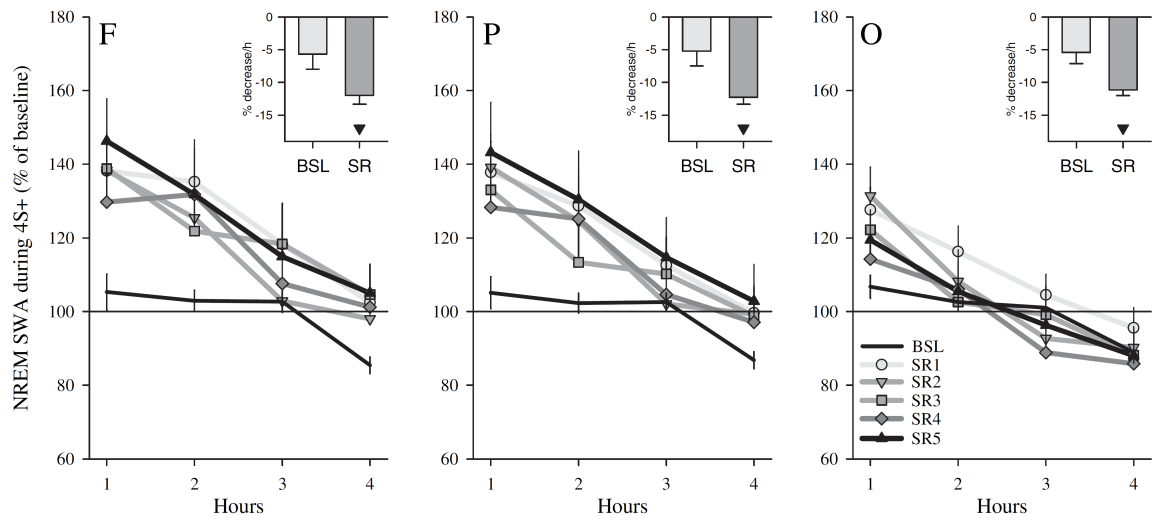


Fig. S3 Time course of SWA in NREM sleep during the 4S⁺ periods during baseline (BSL) and 5 consecutive days of sleep restriction (SR1-5) in the frontal (F), parietal (P), and occipital (O) derivations. Mean hourly values (SEM), $n = 8$. Insets: the rate of decline of SWA (% per 1 h) for BSL and SR days. Mean values (SEM), $n = 8$. Triangles: significant difference from baseline ($P < 0.05$, paired t test).

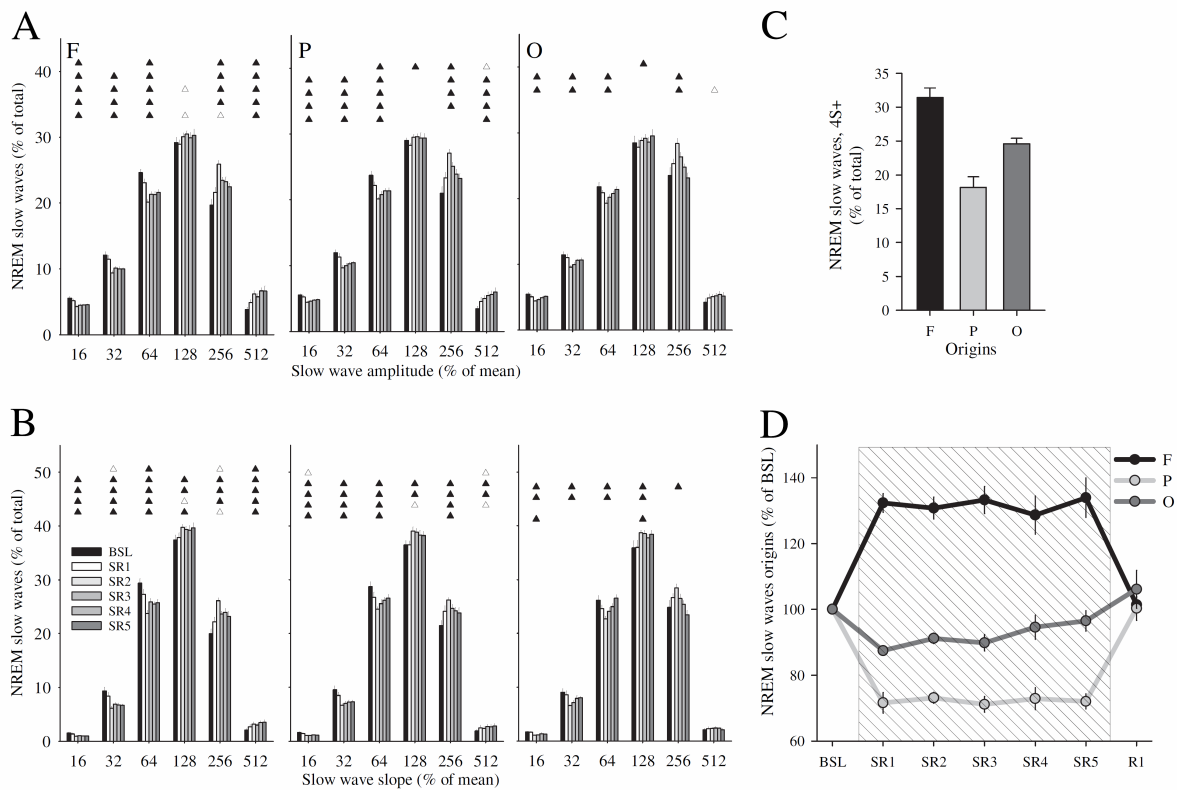


Fig. S4 Histogram of the distribution of NREM sleep slow waves during the 4S⁺ periods as a function of their amplitude (A) or slope (B). The number of slow waves was computed within logarithmically increasing amplitude or slope ranges (% of mean amplitude or slope over all slow waves: 0–16, 17–32, 33–64, 65–128, 129–256, and 257–512%) and plotted against the upper boundary of the corresponding range. Triangles: differences from BSL (black, $P < 0.05$; white, $P < 0.1$). (C) Proportion of slow waves originating at different cortical locations. Mean values (\pm SEM, $n = 6$), expressed as percentage of all slow waves. (D) Number of NREM slow waves originating from the frontal, parietal, or occipital location during the 5 d of SR and recovery (R), plotted as percentage of the corresponding baseline value (BSL; mean values \pm SEM, $n = 6$).

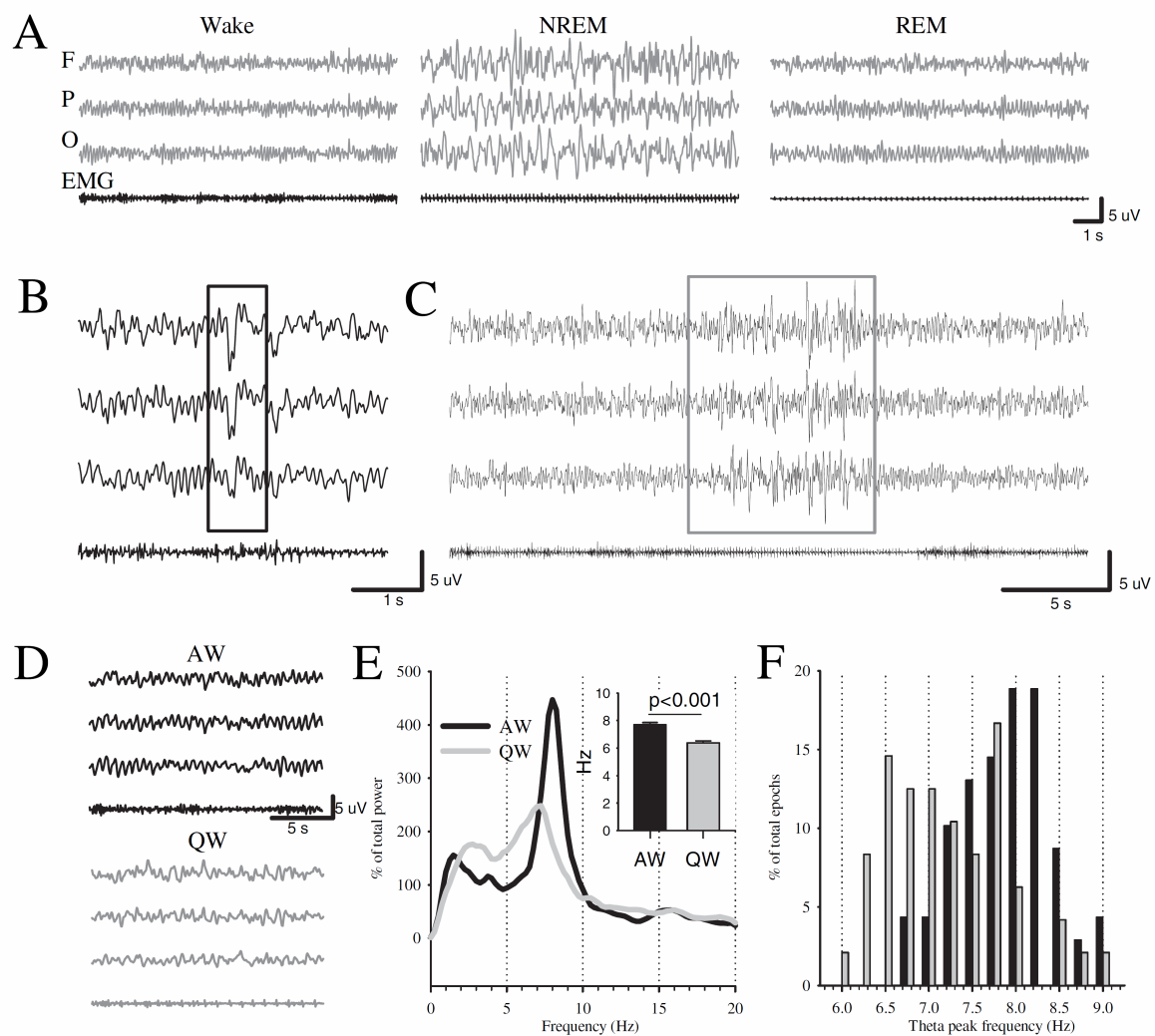


Fig. S5 (A) 12-s EEG traces from frontal (F), parietal (P), and occipital (O) cortex and corresponding electromyogram (EMG) in a representative rat during baseline in wake, NREM sleep, and REM sleep. (B) Isolated slow wave during 20S-. (C) A 30-s recording depicting a typical example of sleep attempt during 20S-: EMG decreases while large slow waves appear in all three derivations. (D) Typical examples of EEG and EMG (Bottom trace) recordings in active waking (AW) and quiet waking (QW): AW is characterized by regular theta activity and high EMG, whereas QW is dominated by a slower EEG pattern and reduced muscle tone. (E) EEG power spectra in AW and QW (~5 min per state) during baseline. Inset depicts mean frequency of the theta peak (\pm SEM). (F). Histogram of the distribution of 4-s epochs in AW and QW as a function of the frequency of the theta peak.

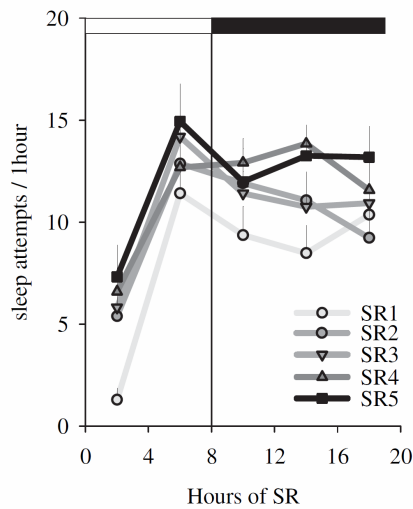


Fig. S6 Time course of the occurrence of sleep attempts (≤ 16 s) during the 20S⁺ periods. Mean 3-h values (SEM), $n = 11$.

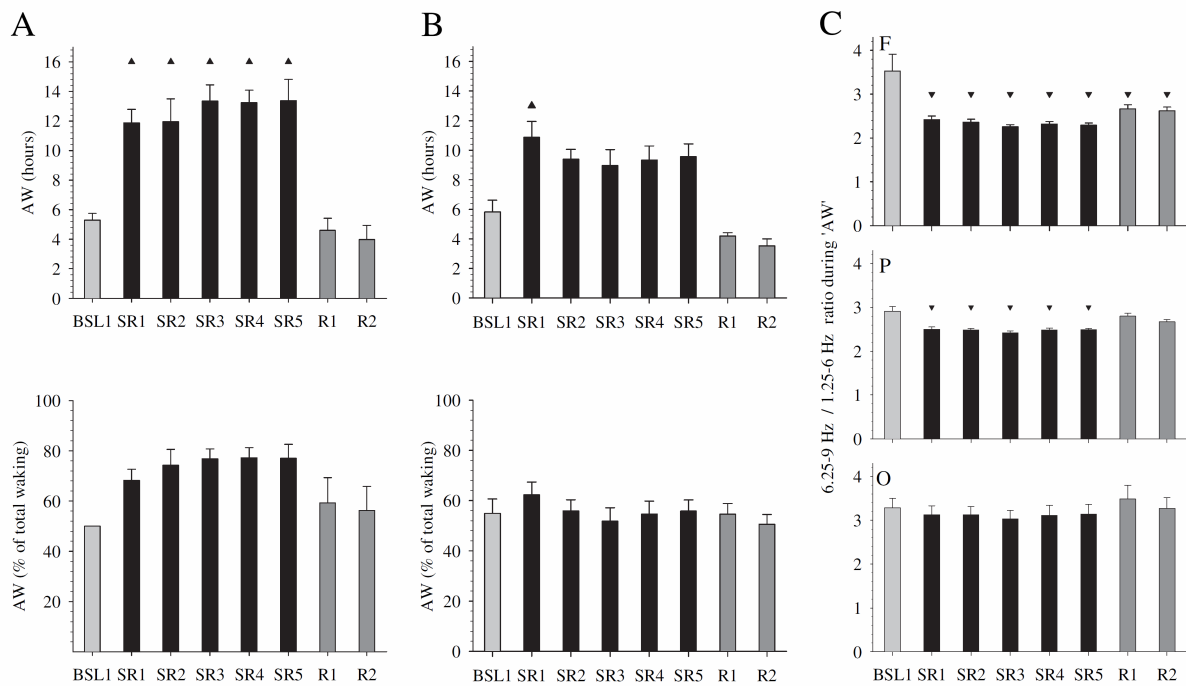


Fig. S7 Effect of disk rotation on the amount of active wake (AW). (A) Amount of AW defined based on the EMG variance: all epochs in which the EMG variance was greater than the median EMG variance during baseline wake were considered AW (waking with high locomotor activity; the remaining epochs were then QW or wake with low locomotor activity). Baseline (BSL), 5 SR days (SR1-5) and 2 d of recovery (R1-2) are shown. Mean values \pm SEM, $n = 6$. By definition, during baseline 50% of waking is AW. Note that the absolute (total in 24 h) amount of AW is significantly higher on SR days (Upper), but the relative amount (% of total wake) is not significantly different between days (Lower, triangles: $P < 0.05$, Dunn-Sidak test). (B) Amount of AW defined based on theta EEG power, which was required to be at least 50% higher than the low frequency EEG power (6.25–9 Hz/1.25–6 Hz > 1.5). Mean values \pm SEM, $n = 6$. The total amount of AW was significantly different from baseline on the first SR day (triangle, $P < 0.05$, Dunn-Sidak test). The relative amount of AW (% of total wake) was not significantly different from baseline. There was high correlation between the amount of AW defined as high EMG and AW defined on the basis of theta activity ($r = 0.68$; $P < 0.001$, Pearson correlation). (C) Mean 6.25–9 Hz/1.25–6 Hz ratio in AW (e.g., epochs with ratio > 1.5) during baseline (BSL), 5 SR d (SR1-5), and 2 d of recovery (R1-2) for the frontal (F), parietal (P), and occipital (O) derivation. Mean values \pm SEM, $n = 8$. Triangles depict days where the ratio was significantly different from baseline (triangles, $P < 0.05$, Dunn-Sidak test).

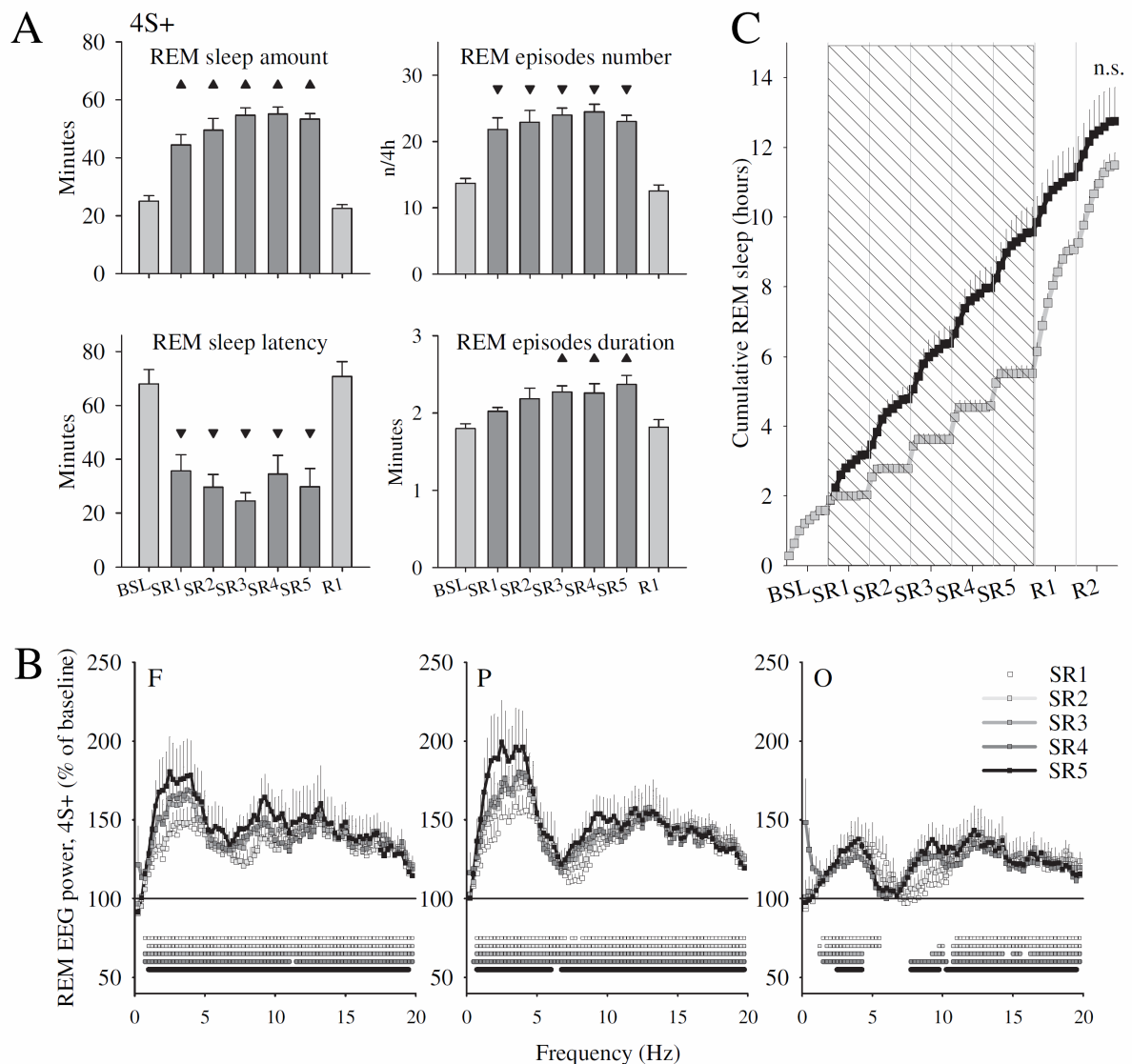


Fig. S8 (A) Mean values (\pm SEM, $n = 11$) of REM sleep amount, REM sleep latency, REM episodes' number and duration during the first 4 h after light onset during baseline (BSL), 5 d of SR (4S+ intervals), and the first day of recovery (R1). Triangles depict significant differences from BSL ($P < 0.05$ after Bonferroni correction, mean \pm SEM, $n = 11$). (B) REM sleep EEG power during 4S+ in the 5 d of SR plotted as percentage of the corresponding baseline period (mean \pm SEM, $n = 8$). Squares depict frequency bins that differed significantly from baseline ($P < 0.05$, paired t test). (C) Cumulative time course of REM sleep during baseline and SR (shaded area). (SEM, $n = 8$). Note that the error bars for the baseline curves increase cumulatively due to the large interindividual variability during the first 24 h of baseline.

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6. Article 2

Sleep EEG changes are related to functional recovery after stroke in the rat

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Submitted

Author contributions: S.L., B.G. and C.L.B designed research; S.L., B.G. and E.C. performed research, J.S. contributed analytic tools; S.L. and B.G. analyzed data; and S.L., B.G. and C.L.B wrote the paper.

Abstract

Study Objectives: Sleep-wake disorders and alterations of sleep EEG are common after ischemic stroke, but their relation to functional recovery remains unclear. We assessed the effects of cortical ischemia on NREM, REM and wake, as well as EEG spectra in those states, in relation to motor function recovery in rats.

Design: We evaluated single pellet reaching (SPR) performance and changes in NREM, REM and wake EEG in both the ipsilateral and contralateral hemisphere during a 30-day recovery period after middle cerebral artery occlusion (MCAo) or sham surgery.

Setting: Basic sleep research laboratory

Patients or Participants: Male Sprague-Dawley rats (n=15)

Interventions: MCAo or sham surgery followed by EEG recordings and SPR tasks

Measurements and Results: Small somatosensory-cortical infarcts ($23.9 \pm 3.1 \text{ mm}^3$), reduce SPR performance and induce widespread changes in EEG spectra in the ipsilateral hemisphere. Post-MCAo recovery of SPR success is negatively correlated to the magnitude of change in ipsilateral power in NREM (7-25Hz) and REM (18-25Hz) and wake (17-21Hz) in the same period. However, power in the same frequency ranges on each experimental day is positively correlated to SPR success (NREM 9-25Hz, REM 13-25Hz).

Conclusions: These results show that EEG power frequencies >7Hz in NREM and REM sleep is involved in motor function recovery after ischemic stroke in rats, suggesting that sufficient desynchronization of the motor cortical network is linked to recovery of motor function after stroke.

Introduction

Sleep-wake disturbances frequently occur in stroke patients, and are linked with poorer recovery of motor and cognitive functions.¹ Sleep plays an important role in learning and memory formation,²⁻³ and is thought to be involved in at least some of the neuronal plasticity necessary for the acquisition of motor skills in healthy animals.⁴⁻⁵ Neuronal plasticity also plays an important role in functional recovery after stroke.⁶⁻⁷ In stroke patients, recovery of cognitive function is related to sleep amount and efficiency,⁸ and learning of motor tasks is sleep dependent.⁹ Moreover, disruption of sleep has detrimental effects on infarct size and expression of plasticity-related genes in rats,¹⁰ while administration of the sleep inducing compound gamma-hydroxybutyrate improved functional recovery in mice.¹¹ Therefore, strategies to improve long-term functional outcome after stroke by targeting sleep might prove to be effective.

In addition to disrupting sleep and wake patterns,^{1, 8, 12-15} stroke alters EEG activity within these states in both patients and animal models; leading to increased theta (6-8Hz)¹³ and delta (1-4Hz) power¹⁶ and reduced beta (15-25Hz) power¹³. Furthermore, reduced spindle activity, related to poor outcome, has been reported in stroke patients.¹⁴ Since slow wave activity (SWA, EEG power between 1-4Hz) during non-rapid eye movement sleep (NREM) is linked to neuronal plasticity and improvements in motor function in the healthy brain,^{4-5, 17} NREM SWA may have a similar beneficial effect on functional recovery after ischemic injury.

Electrophysiological measures (i.e. EEG), give a direct read-out of the state of a brain area in different neurophysiological states (e.g. sleep and wakefulness). Changes in EEG activity therefore directly reflect neuronal plasticity in that area. In the current study we aim to analyze the effects of cortical ischemia on NREM, REM and wake, as well as on the local EEG activity within these states, in relation to recovery of motor function in the rat. Unilateral cortical infarcts were induced in the somatosensory cortex, leaving the motor areas intact.¹⁸ We compared motor cortical EEG activity and forepaw function before stroke and during a 30-day recovery period, resulting in information about the state and structural changes of the motor cortical network, as well as the functional consequences of those changes. Although no structural damage occurs in the motor cortex, its activity is severely disrupted, and extensive remodeling occurs in the recovery period. Changes in EEG activity measured in this area are thus related to plasticity, not loss of cells, and recovery of motor function.

Methods

Animals

15 male Sprague-Dawley rats (7 ischemic, 8 sham, Harlan, Netherlands), weighing 180-200g at the start of the experiment, were used. They lived in individual plexiglass cages on a 12h:12h light-dark cycle. Rats were kept on a feeding schedule throughout the duration of the experiment and received 20-25 grams of chow per day. Neither the feeding schedule, nor the surgeries caused significant weight loss. Chow was presented once per day, either after the single pellet reaching task, or at lights-on. On pellet reaching testing or training days, rats additionally received chocolate flavoured dustless precision pellets (45-mg, product nr. F0299, Bioserve Inc. Frenchtown, NJ, USA). The number of pellets a rat received depended on his reaching success, but did not exceed 50. Water was available *ad libitum* throughout the experiment. All experiments were carried out in the University Hospital Zurich, Switzerland, with governmental approval (Kantones Veterinärämte Zürich, Switzerland) according to local guidelines for the care and use of laboratory animals.

Experimental setup

Rats were implanted with EEG and EMG electrodes after 3 weeks of training on a single pellet reaching task (SPR) (see fig. 1 for an overview). After electrode implantation, they were allowed to recover for one week, during which SPR training continued. 24-hour baseline EEG recordings were then performed, as well as baseline SPR tests.

Focal cortical ischemia by middle cerebral artery occlusion (MCAo) was then induced in the hemisphere contralateral to the rat's preferred paw. Post-MCAo EEG recordings were performed on day 1, 4, 7, 10, 13, 23 and 30. The first post-MCAo EEG recording (D1) was performed ~20h after surgery. Post-MCAo SPR testing was performed on days 2, 5, 8, 11, 14, 24 and 31. Between days 23 and 30, additional daily SPR sessions were introduced as a late-stage means of increasing recovery. Rats were sacrificed after the final SPR session.

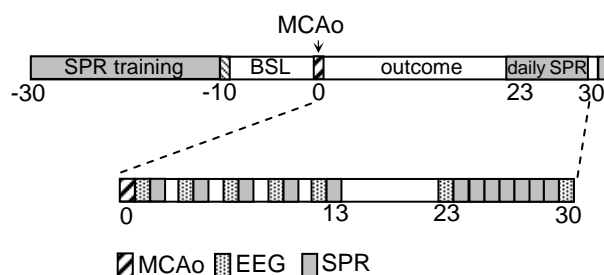


Fig 1: Overview of the time course of the experiment. BSL: baseline; EEG: EEG recording day (dotted); MCAo: MCA-occlusion surgery (striped); SPR: Single pellet reaching (grey).

Single pellet reaching (SPR)

Fine motor skill was assessed using a skilled reaching task (SPR), in which a rat retrieved a small food pellet located outside of the testing cage by reaching for it using one forepaw, as described previously.¹⁸

The rectangular plexiglass SPR testing cage had a shelf mounted 3cm above the cage floor on the outside of each short wall, which could be reached by the rats through a 1.4cm wide slit in the cage wall. Small indentations in the shelves aligned with the edges of the slit assured constant placement of pellets (1.5cm from the inside of the wall).

The first week of three weeks of SPR training consisted of daily 10-minute sessions, in which pellets initially placed within easy reach, but were then moved progressively farther away to encourage use of the forepaw. Once a rat demonstrated paw-preference by making most reaching attempts with a single paw, pellets were only placed in the indentation that could only be reached with that preferred paw. In the second and third week of training, sessions lasted 50 reaching attempts or 15 minutes, whichever occurred first. During these sessions, pellets were presented on alternating sides of the reaching box, so that the rat fully repositioned his body before each reaching attempt and was not randomly reaching through the slot even when no pellets were present.

During SPR baseline and post-MCAo sessions, pellets were only presented on one side of the box, after the rat had moved to the other side. SPR attempts were classified as either successful or failed. In a successful attempt, the rat was able to obtain a pellet in 3 or fewer paw movements and then eat it. SPR success was calculated as the percentage of successfully obtained pellets out of 50 possible attempts. Sessions were videotaped for later verification of the scores.

Surgery

Gold-plated mini screws serving as bipolar epidural EEG electrodes were implanted in the skull over the motor cortex of each hemisphere (rostral electrode: bregma -2mm, 2mm lateral; caudal electrode: bregma +2mm, 2.5mm lateral). Thus, the recorded area included the cortical areas responsible for movement execution, but not the infarct in the somatosensory cortex. Two gold wires inserted in the neck muscles served as EMG electrodes. At least one week after electrode implantation, MCAo was induced by electrocoagulation of the distal part of the middle cerebral artery (MCA), using a method adapted as described previously.¹⁹

In short, the skull overlying the MCA was exposed through an incision in the temporal muscle. A 4-by-5mm window drilled in the frontal bone, exposing the underlying vessels. After carefully removing the dura mater, the MCA and its main branches were closed using bipolar electrocoagulation. The temporal muscle and overlying skin were then sutured back in place. Sham operated rats underwent the same procedure, but without removal of the dura mater and coagulation of the MCA. All rats were lesioned contralaterally to their preferred paw.

For both surgeries, rats were anaesthetized using 2-2.5% isoflurane in 30% oxygen and 70% N₂O. A heating lamp was used to maintain rectal temperature at 35-36°C.

EEG recording and analysis

EEG was recorded at a 100Hz sampling rate, EMG at 200Hz using an Embla A10 amplifier and Somnologica Science software (Embla, Flaga, Iceland). The EEG signal was filtered using a low-cut filter at 0.3Hz. EMG was filtered for 50Hz artefact, and had a low-cut filter at 10Hz.

EEG was visually scored in 8 second epochs based on the EEG signal of the hemisphere contralateral to the MCAo and the EMG (Somnologica Science, Embla, Flaga, Iceland). Wherever the signal contained many artefacts, the signal from the ipsilateral hemisphere was consulted to help identify the vigilance state. Before and after MCAo, wake was characterized by a low amplitude, high frequency EEG pattern and high EMG activity. NREM was characterized by the occurrence of high amplitude slow waves and tonic low EMG activity. The EEG activity during REM resembled that of wake dominated by theta activity, but only occasional twitches were present in the EMG signal. Epochs were classified as belonging to a vigilance state when more than half of an epoch fulfilled the criteria for that state. A total period of 22 hours was scored for each recording day, starting from 1 hour after lights-on. Epochs that included artefacts were excluded from spectral analysis.

Data analysis

Time spent in wake, NREM and REM was calculated for each recording day, as well as the mean episode duration for each of the vigilance states, to assess possible alterations in sleep-wake patterns. Wake and NREM episodes were defined as periods of ≥ 16 s preceded and followed by ≥ 8 s of another vigilance state. REM episodes were defined as being ≥ 8 s long. Power spectra for frequencies from 0.75 to 25Hz were calculated for artefact-free 8s epochs in 0.25Hz bins for each vigilance state using custom routines in Matlab (The Math Works, Inc., Natick, MA, USA). Spectral analysis could not be performed for wake in one MCAo-animal due to a large number of artefacts.

Group and time effects in SPR performance, sleep and wake times and episode duration were analyzed using repeated measures ANOVA (rANOVA). Time effects in power spectra were analyzed separately for each experimental condition by performing rANOVA for each frequency bin, as well as for total power in 4 frequency bands (delta (1-4Hz), theta (6-8Hz), sigma (12-15Hz) and beta (15-25Hz)).

Correlations between spectral changes and outcome (infarct volume or SPR performance) were calculated for each frequency bin, after which frequency ranges of interests were identified as being at least 4 consecutive bins with a significant correlation ($p < 0.1$). Another correlation was then calculated for outcome and average power in that range. Statistical analysis was performed using Matlab and SPSS (IBM, Somers, NY, USA). Results were considered significant when $p < 0.05$ unless indicated otherwise.

Histology

Rats were killed with an overdose of sodium pentobarbital and perfused transcardially with 0.1M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 0.1M PBS. Brains were removed, post-fixated in 4% PFA in 0.1M PBS and cryoprotected in 30% sucrose in 0.1M PBS at 4°C. 30µm thick coronal sections were collected and stained using cresyl violet. Infarct volume was calculated based on the distance between sections and the surface area of sections as measured using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA).

Results

Ischemic lesions

Analysis of brain sections showed that MCAo resulted in infarcts located in the somatosensory cortex. Infarct volume was $23.9 \pm 3.1 \text{ mm}^3$ (mean \pm s.e.m). Ischemic damage did not extend into subcortical regions, nor into the motor cortex (fig. 2).

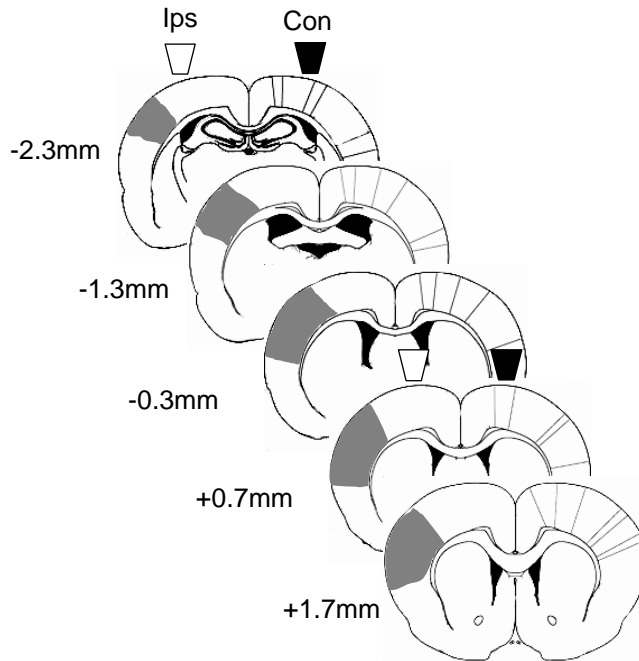


Fig 2: Schematic drawing showing infarct and EEG electrode positions.
Ips: electrodes recording ipsilateral hemisphere, *Con:* electrodes recording contralateral EEG trace.

Pellet reaching

SPR success was markedly reduced after MCAo, by on average 60% (fig. 3) (rANOVA factor group: $F(3.4)=4.1$, $p=0.008$), time ($F(8)=2.899$, $p=0.008$), time*group interaction ($F(8)=5.002$, $p<0.001$). Performance slowly improved and returned to baseline levels on day 31.

The improvement in SPR after the daily rehabilitative SPR sessions between days 23 and 31 was not different from the spontaneous improvement in the preceding 10-day period ($16.7 \pm 9.2\%$ from day 23 to 31, and $14.7 \pm 8.9\%$ from day 13 to 23, mean \pm s.e.m), indicating that this late-phase intervention had limited benefit in increasing recovery speed.

The acute reduction of SPR success from baseline was significantly correlated to infarct volume ($R=-0.713$, $p=0.036$), whereas the amount of subsequent recovery between days 2 and 31 was not ($R=0.384$, $p=0.198$). However, rats with smaller infarct volumes tended to show the first SPR improvement sooner than those with larger volumes ($R=0.661$, $p=0.053$).

Sham surgery had no effect on SPR success, and rehabilitative SPR sessions did not alter SPR success in this group.

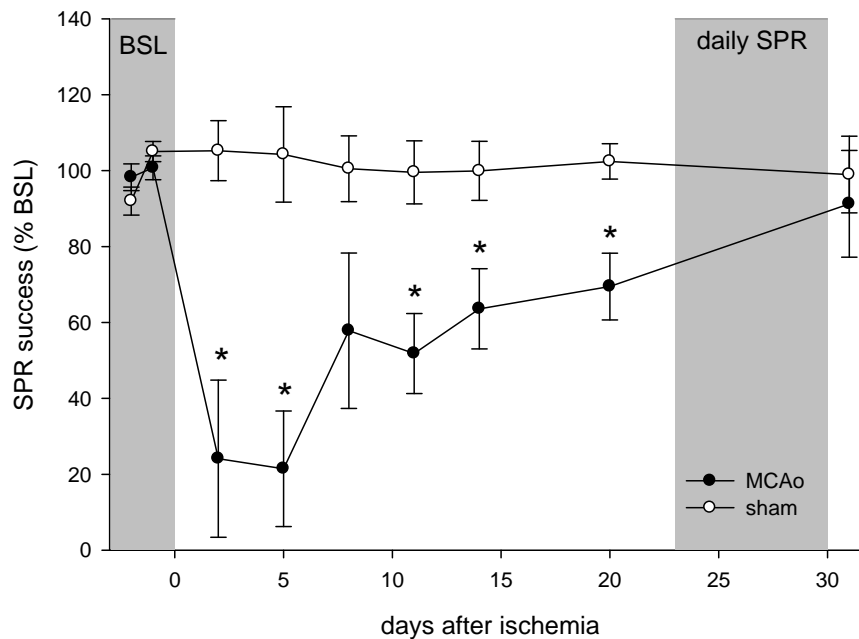


Fig 3: Pellet reaching success in MCAo (black circles) and sham-operated animals (white circles) is shown as a percentage of average performance on the two baseline days. * $p < 0.05$ in t -test compared to sham-animals after significant group effect in r ANOVA. Values are mean \pm s.e.m.

			BSL	D1	D4	D7	D10	D13	D23	D30
NREM	L	MCAo	41.5 \pm 2.3	38.4 \pm 5.9	45.3 \pm 3.2	38.5 \pm 1.8	38.2 \pm 1.6	35.8 \pm 1.6 ^b	37.5 \pm 1.6	42.2 \pm 0.8 ^a
		sham	42.5 \pm 2	42.9 \pm 1.7	43.3 \pm 2.6	37.8 \pm 0.1	48.7 \pm 3.9	45.6 \pm 2.7	44.1 \pm 2.3	56.2 \pm 0.4 ^b
	D	MCAo	39 \pm 1.8	43.4 \pm 0.8	47.5 \pm 1.8	38.2 \pm 2.1	37.9 \pm 2.2	35.1 \pm 2.3	38.4 \pm 2	40.5 \pm 1.1
		sham	39.7 \pm 1.2	42.6 \pm 2.8	40.9 \pm 2.2	43 \pm 0.1	36.9 \pm 1.7	37.2 \pm 1.9	39.2 \pm 1.7	40.3 \pm 1.3
REM	L	MCAo	6.6 \pm 1	2.2 \pm 0.8 ^{a,b}	4.5 \pm 0.7	4.8 \pm 0.6	6.8 \pm 0.7	5.9 \pm 0.5	5.7 \pm 0.7	5.3 \pm 0.5
		sham	5.6 \pm 0.5	6.2 \pm 0.5	6.7 \pm 0.5	6.7 \pm 0.4	8 \pm 0.8	7.4 \pm 0.3 ^b	6 \pm 0.6	6.8 \pm 0.4
	D	MCAo	10 \pm 1.1	10.5 \pm 0.9	10.1 \pm 0.5	10.5 \pm 0.4	10.2 \pm 0.3	9.3 \pm 0.2	10.3 \pm 0.4	11.6 \pm 0.7
		sham	10.9 \pm 0.5	8.6 \pm 0.4	9.7 \pm 0.7	11.5 \pm 0.3	8.5 \pm 0.6	8.8 \pm 0.5	10 \pm 0.5	9.8 \pm 0.4
Wake	L	MCAo	51.9 \pm 2.4	59.4 \pm 5.9	50.2 \pm 3.5	56.7 \pm 2.1	54.9 \pm 1.9	58.3 \pm 1.5 ^{a,b}	56.8 \pm 1.6	52.5 \pm 1.1 ^a
		sham	51.9 \pm 2.4	50.9 \pm 1.4	50 \pm 2.6	55.5 \pm 0.3	43.3 \pm 4.4	47 \pm 2.9 ^b	49.9 \pm 2	37 \pm 0.8 ^b
	D	MCAo	51 \pm 2.1	46.1 \pm 1.1	42.4 \pm 1.8	51.3 \pm 2.4	51.9 \pm 2.2	55.6 \pm 2.3	51.3 \pm 2	47.9 \pm 1.5
		sham	49.4 \pm 1.3	48.8 \pm 2.6	49.4 \pm 1.7	45.5 \pm 0.4	54.5 \pm 1.6	54 \pm 2.1	50.8 \pm 2	50 \pm 1.7

Table 1: Times spent in wake, NREM or REM as percentage of recording time in the light (L) and dark (D) period. Letters indicate difference between MCAo and sham (^a) or difference from BSL (^b) ($p < 0.05$ in t -test after significance in r ANOVA, after Bonferroni-correction). All values are mean \pm s.e.m.

Vigilance states

We did not find any major changes in the amount of NREM, nor in time spent awake after MCAo (Table 1). Time spent in REM during the light phase was acutely reduced by ~35% after MCAo, and returned to sham and baseline levels on D4. However, total wake, NREM and REM time, as well as wake, NREM and REM in the dark phase and NREM and wake in

the light phase, were not affected by surgery. Episode duration (Table 2) remained unaltered in MCAo and sham animals.

			BSL	D1	D4	D7	D10	D13	D23	D30
NREM	Light	MCAo	99±13	113±13	136±17	99±9	93±6	99±12	97±14	120±5
		sham	109±11	118±10	115±11	121±1	128±16	122±12	115±11	143±8
	Dark	MCAo	114±8	116±8	114±11	116±10	102±14	105±11	111±9	125±5
		sham	114±5	117±9	133±10	111±5	113±8	107±6	103±11	120±3
REM	Light	MCAo	56±10	51±13	79±5	76±8	76±5	74±8	65±9	75±3
		sham	82±6	91±8	89±10	71±1	83±5	84±4	76±4	73±6
	Dark	MCAo	76±8	76±15	90±7	100±6	90±8	84±4	86±8	83±3
		sham	86±4	71±6	82±6	83±4	73±7	77±5	88±4	83±3
Wake	Light	MCAo	124±8	193±40	147±22	142±7 ^a	133±15	154±12	140±14	143±8 ^a
		sham	125±6	132±4	129±10	174±2	105±13	119±10	122±4	84±3
	Dark	MCAo	149±14	125±11	117±8	144±6	143±5	165±7	136±6	137±7
		sham	139±9	128±9	131±4	120±6	152±10	152±7	138±7	159±6

Table 2: Episode duration in wake, NREM or REM in the light and dark period. Letters indicate difference between MCAo and sham (^a) or difference from BSL (^b) ($p < 0.05$ in t-test after significance in rANOVA, after Bonferroni-correction). All values are mean \pm s.e.m.

EEG power spectra

Although MCAo did not have a major effect on NREM, REM and wake amounts and episode durations (table 1 and 2), EEG activity within these states was severely altered by the infarct. The hemisphere ipsilateral to the infarct was most obviously affected, resulting in a marked asymmetry in the EEG (fig. 4), although vigilance states could still

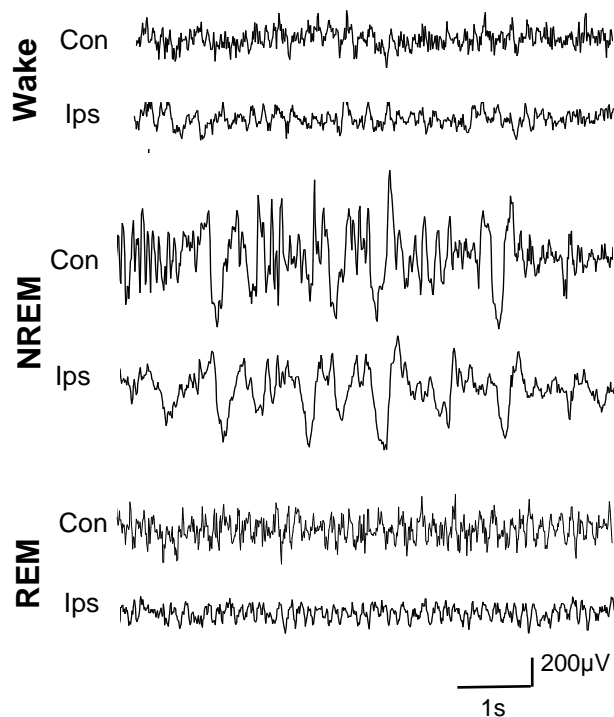


Fig 4: Representative examples of the EEG and EMG signal in wake, NREM and REM in the ipsilateral (Ips) and contralateral (Con) hemisphere on D4 after MCAo.

readily be distinguished.

rANOVA revealed significant time effects in EEG power after MCAo in a wide range of frequencies in the ipsilateral hemisphere in wake (1-3Hz and 6-25Hz, N=6, fig.5), NREM (1-25Hz, N=7, fig.6) and REM (1-5.5Hz and 7-25Hz, N=7, fig 7.), and contralateral in NREM (fig.6). Sham surgery had no effect on spectral power (N=8, fig. S1).

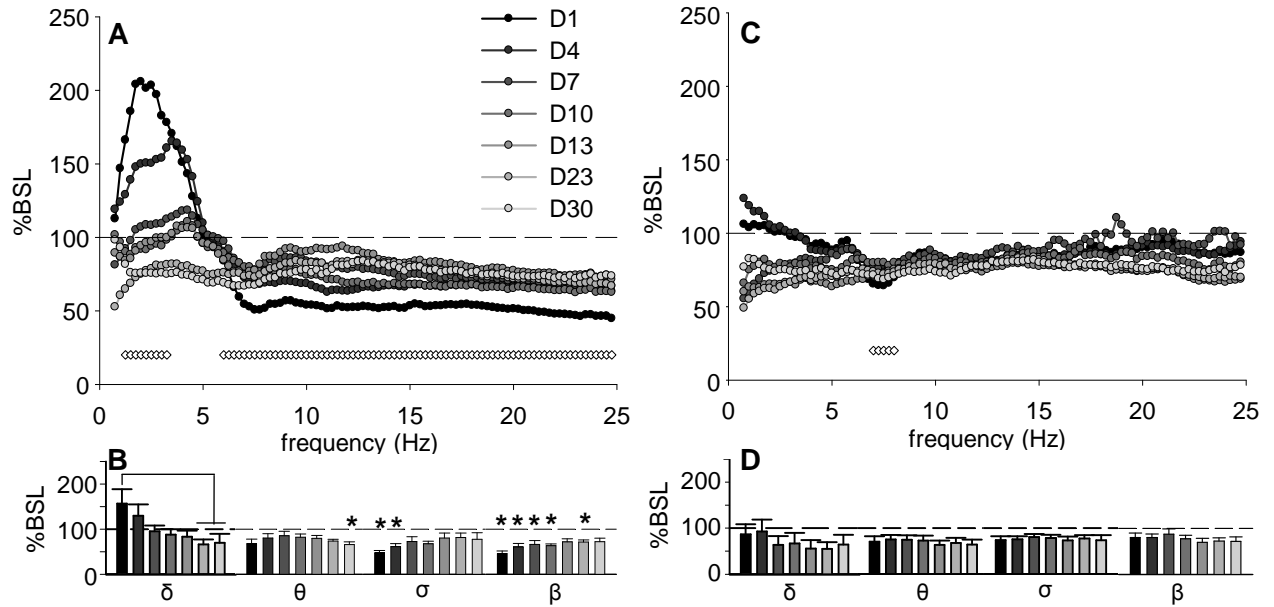


Fig 5: Average wake power spectra as percentage of BSL for post-MCAo days in the ipsilateral (A,B) and contralateral (C,D) hemisphere. Diamonds under the curves indicate a significant time-effect in rANOVA ($p<0.05$). Power in delta (1-4Hz), theta (5-8Hz), sigma (12-15Hz) and beta (15-25Hz) frequency bands is displayed in the bar plots (mean \pm s.e.m). Asterisks indicate a significant difference from 100% BSL ($p<0.05$). Brackets indicate significant differences between days (Tukey's HSD, $p<0.05$).

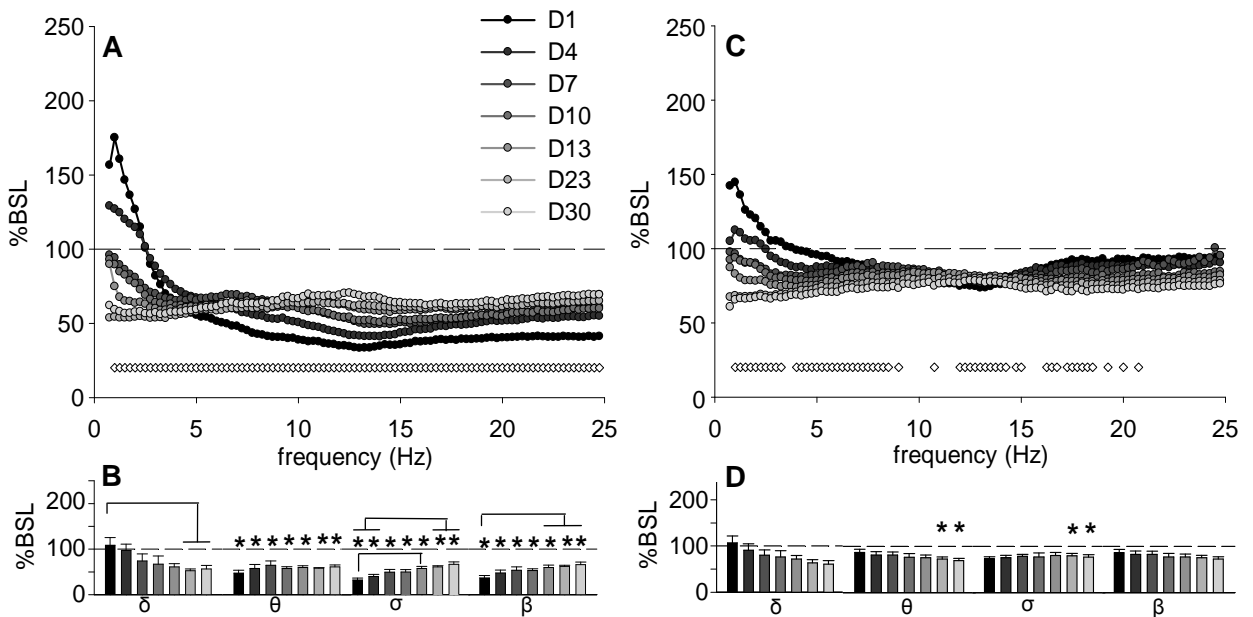


Fig 6: Average NREM power spectra as percentage of BSL for post-MCAo days in the ipsilateral (A,B) and contralateral (C,D) hemisphere. Diamonds under the curves indicate a significant time-effect in rANOVA ($p<0.05$). Power for delta (1-4Hz), theta (5-8Hz), sigma (12-15Hz) and beta (15-25Hz) frequency bands is displayed in the bar plots (mean \pm s.e.m). Asterisks indicate a significant difference from 100% BSL ($p<0.05$). Brackets indicate significant differences between days (Tukey's HSD, $p<0.05$).

Post-hoc analysis of whole spectra and spectral bands showed that wake EEG power in frequencies >6Hz was significantly reduced on D1 and D4 compared to BSL (100%) and later days. Power between 8-15Hz returned to baseline levels on D7, but activity in the theta (5.5-8Hz) and beta range (15-25Hz) remained below BSL and did not change significantly during the rest of the experiment. A similar effect was found in the ipsilateral NREM spectra, where power >6Hz was significantly reduced from BSL on D1 (by ~70%). Power in this entire range (6-25Hz) showed a significant recovery from D1 to D7, but remained stable below BSL after that. Ipsilateral spectral power in REM also showed an acute reduction from BSL and later days in frequencies >7Hz on D1 and D4. Power increased between D4 and D7, and remained stable below BSL after that.

In all vigilance states, power in the delta frequency range was acutely increased after MCAo (1-5Hz in wake and REM, 1-3Hz in NREM). Although this response was highly variable, and power did not significantly differ from BSL (100%) on D1 and D4 in wake and NREM, it was significantly elevated compared to D7 and later days in all states. A similar, but much smaller increase was present on D1 in the sham group, indicating that this may in part be an effect of surgery, rather than only ischemia alone.

The only spectral changes in the contralateral hemisphere were found in NREM. These were much smaller and less widespread than the ipsilateral changes, and consisted of an acute reduction in power from 7-16Hz on D1, after which power in both higher and lower frequencies is also reduced until power is below BSL levels in the entire frequency range on D30, although spectra did not differ significantly from one another after D4.

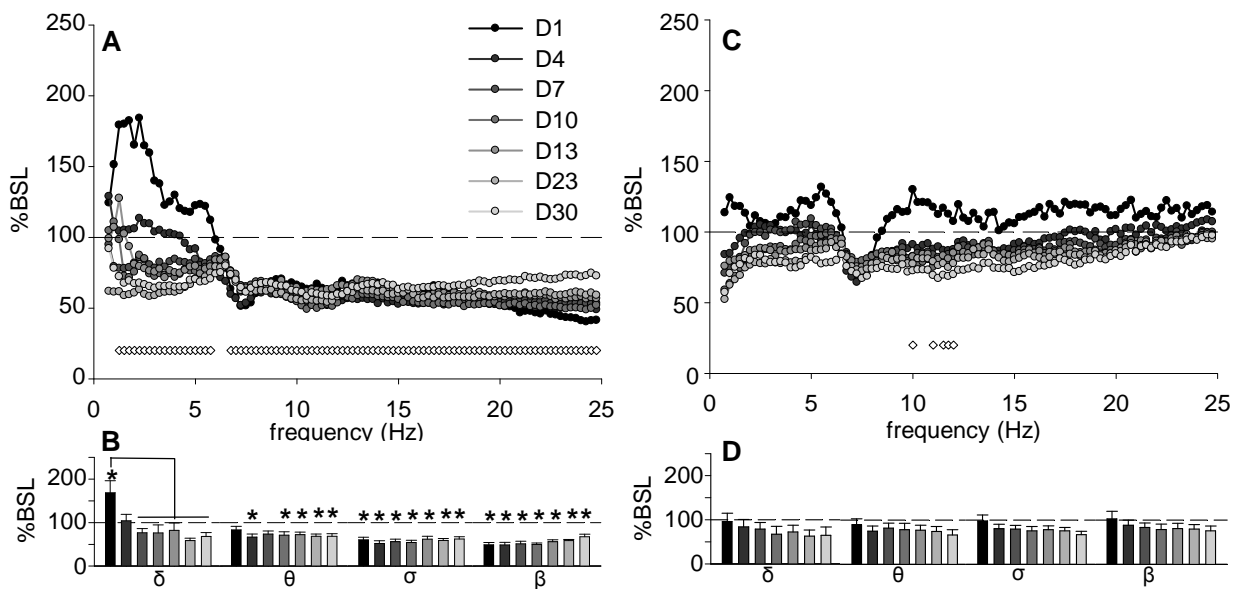


Fig 7: Average REM power spectra as percentage of BSL for post-MCAo days in the ipsilateral (A,B) and contralateral (C,D) hemisphere. Diamonds under the curves indicate a significant time-effect in rANOVA ($p < 0.05$). Power for delta (1-4Hz), theta (5-8Hz), sigma (12-15Hz) and beta (15-25Hz) frequency bands is displayed in the bar plots (mean \pm s.e.m). Asterisks indicate a significant difference from 100% BSL ($p < 0.05$). Brackets indicate significant differences between days (Tukey's HSD, $p < 0.05$).

Changes in EEG and behavioral outcome

To investigate a possible link between EEG changes and recovery of motor function, we performed a linear correlation analysis between EEG power and SPR success.

Are the changes in SPR related to changes in the EEG? The acute SPR deficit ($\Delta D2$ -BSL) was not significantly correlated to acute EEG changes ($\Delta D1$ -BSL) in NREM or REM, but showed a tendency towards a negative correlation in wake (10-14Hz, $r=-0.87$, $p<0.1$). Recovery of post-MCAo SPR success ($\Delta D31$ -2) was negatively correlated to changes in ipsilateral beta power during that period ($\Delta D30$ -1) in wake (17-21Hz, $r=-0.96$, $p<0.05$) and REM (18-25Hz, $r=-0.94$, $p<0.05$), and to alpha and sigma power in NREM (7-15Hz, $r=-0.94$, $p<0.05$) (fig. 8A). Thus, although acute SPR impairment was not closely related to acute EEG changes, animals that show much post-MCAo improvement show little change in their EEG spectra. However, SPR success on each day is positively correlated to power in the higher frequency ranges in NREM (9-25Hz), REM (13-25Hz) and negatively correlated to power in the delta frequency range in wake (1-5Hz) and REM (1-6Hz) (fig. 8B).

Can acute changes predict recovery? The amount of SPR improvement after ischemia ($\Delta D31$ -2) is not significantly correlated to the acute changes in the EEG spectrum ($\Delta D1$ -BSL). The total post-MCAo ($\Delta D30$ -1) change in ipsilateral power in NREM (4.5-7Hz, $r=-0.9$, $p<0.05$ and 9.5-25Hz, $r=-0.9$, $p<0.1$), however, was positively correlated to the size of the initial SPR deficit ($\Delta D2$ -BSL). Thus, although acute EEG changes were a poor predictor for later functional recovery, animals that show a large initial SPR deficit show large changes in their EEG during recovery. The initial SPR deficit ($\Delta D2$ -BSL) is negatively correlated to post-MCAo SPR recovery ($\Delta D31$ -2, $R=-0.7$, $p=0.04$). Animals that showed the largest improvements in SPR were also the ones with the smallest initial deficit, and the smaller post-MCAo EEG changes.

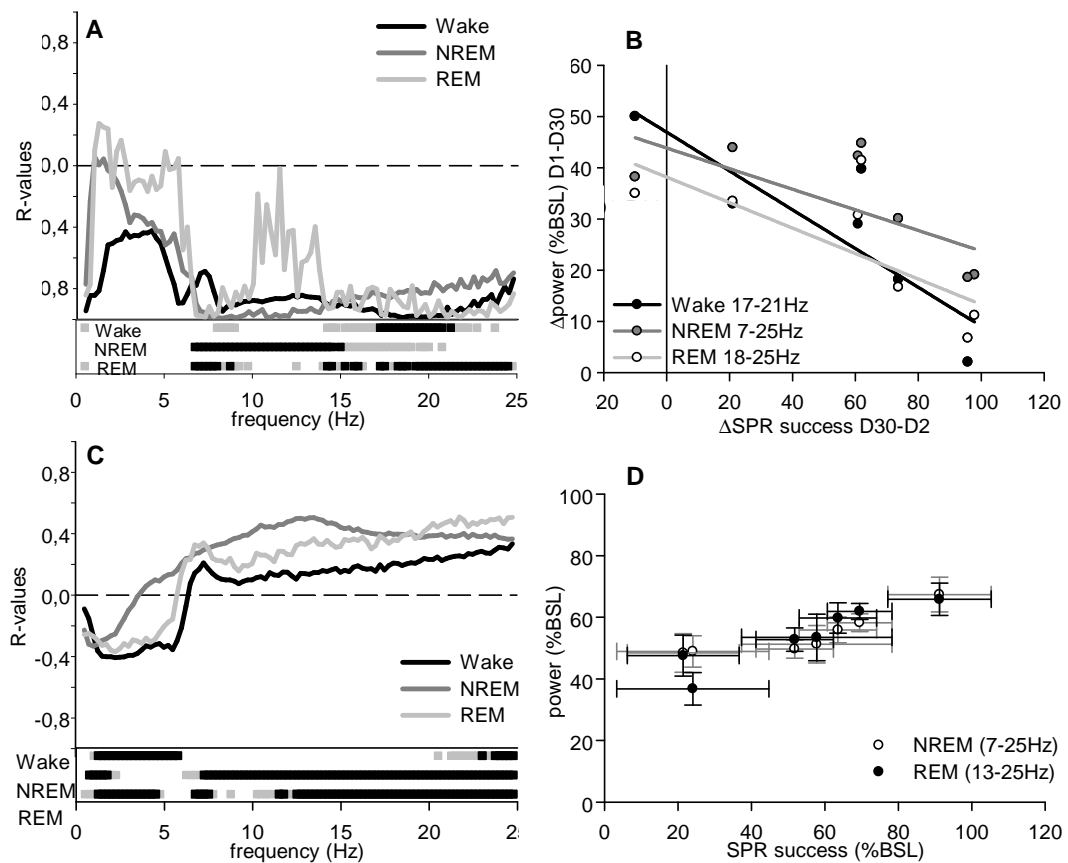


Fig 8: A, left: R-values of correlation between recovery of post-MCAo SPR performance and change in spectral power per bin for each vigilance state. Blocks below the curves indicate significance (black: $p < 0.05$; white: $P < 0.1$). Right: correlation between change in SPR performance and average power within a significant frequency range. B: left: R-values of correlation between SPR performance and spectral power per bin for each vigilance state. Blocks below the curves indicate significance (black: $p < 0.05$; white: $P < 0.1$). Right: SPR performance and power in the significant frequency ranges. Values are mean \pm s.e.m for each experimental day.

Discussion

In the current study we analyzed the effects of cortical ischemia on wakefulness, NREM and REM sleep, as well as on local cortical EEG activity within these states, and their relation to motor function recovery in the rat. By recording EEG in a cortical area that is not structurally damaged by the MCAo, we were able to get an electrophysiological read out of the functional remodeling there, without the confounding effects of massive cell death occurring in the infarcted area.

MCAo reduced performance on the SPR task, which gradually improved during a 30-day recovery period. However, in contrast with previous studies in rodents,^{10, 13} we did not find any major changes in the amount of sleep and wakefulness, nor in episode duration. This is likely due to our much smaller infarcts, which cause mostly changes that are only visible in the local EEG, not in the global vigilance states.

EEG power in frequencies above ~7Hz was acutely reduced in the hemisphere ipsilateral to the infarct in all vigilance states. Contralateral spectra in wake and REM are not affected by MCAo, but power in the theta range was reduced in NREM on the first 4 days after ischemia. Thus, the bilateral reduction in NREM theta and sigma power seems to be a state-specific occurrence, whereas the widespread ipsilateral power reductions are not, even though they are largest during sleep.

The suppression of the faster part of the EEG spectrum by an ischemic lesion is in line with previous results in mice.¹³ Similar to our results, spindle activity in the ischemic hemisphere is reduced in the acute phase after stroke in patients, and recovered in the chronic phase.¹⁴ Sigma band power and spindle activity in NREM are involved in learning processes in healthy subjects,²⁰ as is fast EEG activity;²¹ their role in the relearning of motor skills after stroke may be similar.

Learning of a skilled reaching task causes increased motor cortical SWA in NREM shortly after learning⁴ and improvement on the task was correlated to the amount of NREM SWA. Although MCAo acutely causes a highly variable SWA increase in all vigilance states, we did not find a correlation between the amount of recovery and changes in NREM SWA. Large, long-term changes in SWA are thus unlikely responsible for the plasticity resulting in long-term functional recovery in the ischemic brain. However, we recorded EEG on the day preceding the reaching task, and not after learning. Any short term increases in SWA caused by the reaching would therefore not be recorded. We did, however, find that daily performance was negatively correlated to SWA in wake and REM.

Apart for widespread changes in the damaged hemisphere, MCAo caused changes in the power spectrum of the undamaged hemisphere only in NREM. Stroke is known to induce activation of the corresponding undamaged brain area and functional recovery is associated with a recovery of interhemispheric functional connectivity.²² Similar to our results, changes in spindle activity were present in both hemispheres after stroke in patients, and suggest further interhemispheric interplay.^{14, 23} However, contralateral EEG changes in NREM and other vigilance states were not correlated to functional recovery, and frequency ranges associated with recovery in the ipsilateral hemisphere, were unaffected contralaterally.

We found that the amount of post-MCAo motor function improvement was negatively correlated the magnitude of the EEG change the beta frequency range in wake (17-21Hz) and REM (18-25Hz) and in NREM (7-20Hz), although SPR performance on each experimental day is positively correlated to EEG power in the same ranges in NREM and REM. Animals with large initial motor deficits showed the largest amount of recovery of EEG power, although their total amount of motor function recovery is less than that of animals with a smaller initial deficit. Small post-MCAo EEG changes indicate limited disruption of motor cortical activity, allowing faster relearning of old motor skills, or finding alternate strategies. Loss of high frequency EEG power reflects a decreased ability of the recorded motor area to show desynchronized activity, reducing its ability to form complex activation patterns necessary for the execution of fine motor skills.

The loss of power in the wide range of higher EEG frequencies may be the result of excessive tonic inhibition,²⁴ as it is present in all vigilance states in the first week after MCAo.

Since desynchronization of the motor cortical network seems to be related to the recovery of motor function after stroke, increasing the ability of the cortex to desynchronize may be effective in improving the amount and rate of recovery. Desynchronization may be increased by increasing neuronal activity (for example by application of stimulants such as amphetamine).²⁵⁻²⁶ Conversely, desynchronization may be increased by reducing neuronal inhibition.²⁴ Both options have been shown to be effective in promoting motor function recovery, although in both cases timing is essential, as premature application would exacerbate excitotoxicity.

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Supplementary Figure

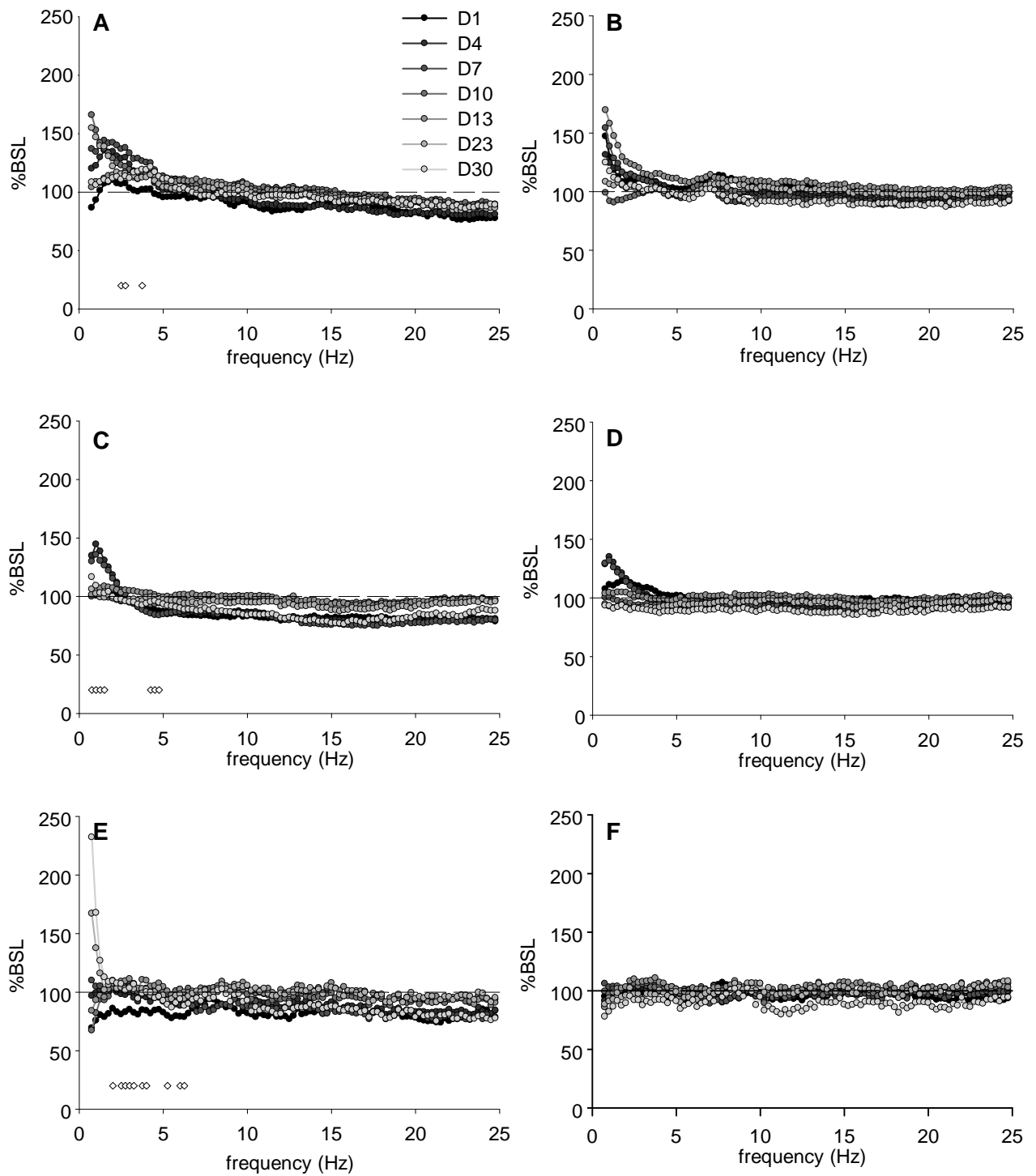


Fig S1: Average power spectra as percentage of baseline in sham-operated rats for post-surgery days in the ipsilateral (A,C,E) and contralateral (B,D,F) hemisphere, in wake (A,B), NREM (C,D) and REM (E,F). Diamonds under the curves indicate a significant time-effect in rANOVA ($p < 0.05$).

7. General Conclusion and Outlook

7.1 Sleep regulation and chronic sleep loss

It is well established that sleep is homeostatically regulated on the short term, but data on sleep regulation is less clear when wakefulness is extended for a longer period of time. Long term sleep deprivation and chronic sleep restriction in humans and rats did not always result in the characteristic SWA rebound indicative of increased sleep pressure. Indeed, results by Kim and colleagues (2007) indicated that rats either stopped regulation their SWA, or reset their homeostatic setpoint. However, a recent SR experiment showed that humans do not reset their homeostat, and continue to show markers of increased sleep pressure (Akerstedt et al. 2009).

These results have a number of implications: firstly, they may indicate that with some effort, rats and humans may be able to learn to live with very little sleep. Secondly, this discrepancy between the effects of chronic sleep restriction on sleep homeostasis in rats and humans has implications for the validity of the rat as an animal model for investigating the effects of chronic sleep loss. And thirdly, these results suggest that the 2-process model of sleep regulation may not apply in chronic conditions in rats, but does apply in humans. The 2-process model of sleep regulation is one of the cornerstones of sleep research, and has been shown to apply in many species in conditions of spontaneous and enforced wakefulness, as well as extension of sleep. Such a striking species difference would affect the validity of the model, as well as the interpretation of previously obtained data.

In the first article presented in the current thesis, we used more refined methods than previous studies to investigate the effects of chronic sleep loss on both sleep and wakefulness during and after 5 days of SR (i.e. stricter enforcement of wakefulness and continuous high-quality EEG recordings in three different cortical regions).

In contrast to short term sleep deprivation methods, where animals can be kept awake by gentle handling (e.g. presenting them with novel objects to explore), chronic sleep deprivation relies on automatic sleep deprivation methods. In this study we used the disc over water (DOW), which promotes wakefulness by forced locomotion. Additionally, we observed the animals 24 h/d and presented extra stimuli as soon as the EEG or behavior showed any signs of sleep. These extra measures were needed to prevent an increasing number of short sleep episodes, indicating that traditional methods of automatic “total” sleep deprivation, which use a fixed rate of perturbation, are insufficient to enforce chronic wakefulness. However, even with extra stimulation, it proved impossible to keep the animals awake for more than 90-95% of the time.

The number of sleep attempts rose rapidly during SR, indicating an increased sleep propensity, which was associated with higher SWA, and thus elevated sleep pressure. Moreover, we quantified SWA during both sleep and wakefulness.

In the 2-process model of sleep regulation, sleep pressure increases during periods of wakefulness, and decreases again during sleep. Thus, it is usually interpreted with the assumption that no dissipation of sleep pressure occurs during wakefulness, and

consequently only SWA occurring during (NREM) sleep is analyzed in both short- and long-term sleep deprivation studies.

We found that SWA and theta power (5-7Hz) were increased during wakefulness during SR. Moreover, individual slow waves could be observed in the wake EEG, when animals showed locomotor activity and had their eyes open. This leakage of sleep-like activity into wakefulness has not been considered in previous studies. We show that SWA during short sleep attempts, as well as SWA during wakefulness compensates part of the accumulated sleep pressure. This may account for the lack of homeostatic increase in SWA observed during sleep opportunities by Kim and colleagues, who interpret this as an allostatic response. Additionally, the rate of SWA accumulation remained stable throughout SR, further supporting the preservation of sleep homeostasis during chronic SR.

Thus, we show that sleep homeostasis as described by the 2-process model of sleep regulation is intact under chronic sleep loss conditions, and shows many of the same markers as acute sleep loss, although we cannot rule out that even longer periods of sleep loss produce different results. Our current results allow for the extrapolation of results from short term sleep loss experiments to long term experiments. Moreover, it shows that sleep regulation under chronic conditions is not essentially different from short-term regulation.

So far, no other animal studies and only one human study (Goel et al. 2009) have shown regional differences in responses to chronic sleep loss. In our study, we found that the frontal and parietal cortex showed increased SWA during NREM sleep during the sleep opportunities in response to SR, but the occipital cortex did not. However, the occipital cortex showed increased SWA during wakefulness in the 20-hour periods of forced locomotion, whereas the frontal and parietal cortex did not. The number of slow waves originating in the frontal cortex was also increased during SR, while it was decreased in the occipital cortex.

During chronic sleep loss, the brain tries to dissipate sleep pressure in every possible way, even during wakefulness and REM sleep, although most of the recovery (~90%) occurs during NREM sleep. The leakage of sleep-like EEG activity into wakefulness and its contribution to the dissipation of sleep pressure shows that although SWA is a valid marker for sleep homeostasis, a strict dichotomy between sleep and wakefulness may be too simple. Therefore, it is essential to include analysis of the wake EEG to understand the homeostatic response, particularly when wakefulness is prolonged for many hours or days.

Even when using very short epoch lengths of a few seconds to define sleep and wake episodes, transitions between states do not occur simultaneously in all brain areas, and individual slow waves may occur not only in an electrophysiological wake-context, but also in a behavioral one. Such relatively small events may go unnoticed when using power spectral density, the current golden standard of sleep EEG analysis. Therefore, analysis of individual slow waves, whose slope and amplitude are sensitive indicators of sleep pressure, may be more suitable for analyzing sleep homeostasis in the EEG or LFP, when combined with detailed observations of behavior. Movement artifacts in the wake EEG

may be removed using methods like template matching and independent component analysis, to allow for better quantification of slow wave activity in wakefulness.

There are likely species differences in the extent of sleep-leakage. Rats may be more likely to show intrusions of sleep into wakefulness than humans, because of their natural polyphasic sleep patterns and faster switching between vigilance states, whereas human may show lapses in attention accompanied by some slowing of the EEG, rather than a full-blown slow wave.

From a functional point of view, an intriguing possibility is that “wake slow waves” may decrease sleep need because they provide at least some of the benefits of sleep slow waves at the cellular level. Although highly speculative at this time, this hypothesis can be tested experimentally, for instance by measuring whether molecular and electrophysiological markers of synaptic strength, which decrease during sleep (Vyazovskiy et al. 2008a), are also decreased during wake with sustained increase in SWA. It is worth remembering, however, that an increase in wake SWA, whether caused by prolonged wake or by certain drugs (e.g., anticholinergic agents), consistently results in cognitive deficits (Van Dongen et al. 2003; Cirelli et al. 2008; Meerlo et al. 2008).

7.2 EEG changes and functional recovery after stroke

Sleep disorders frequently occur in stroke patients, and are linked with a poorer recovery of motor and cognitive functions (Bassetti 2005, 2011). Sleep plays an important role in learning and memory formation (Diekelmann et al. 2010), and is thought to be involved in at least some of the neuronal plasticity necessary for the acquisition of motor skills in healthy animals. Such neuronal plasticity also plays an important role in functional recovery after stroke (Cheatwood et al. 2008; Diekelmann et al. 2010). Therefore, sleep may affect functional recovery after stroke.

To gain insight in the relation between sleep and functional recovery, we analyzed NREM sleep, REM sleep and wakefulness, as well as local EEG activity within these states in the ipsilateral and contralateral hemisphere, and performance on a skilled reaching task during a 30-day recovery period after MCAo in the rat.

The unilateral cortical infarcts induced using this stroke model were located in the somatosensory cortex, leaving the motor areas intact (Gharbawie et al. 2005), current thesis). We compared motor cortical EEG activity and forepaw function before stroke and during recovery period, resulting in information about the state and structural changes of the motor cortical network, as well as the functional consequences of those changes. Although no structural damage occurs in the motor cortex, its activity is severely disrupted, and extensive neuronal remodeling occurs in the recovery period. Changes in EEG activity measured in this area are thus likely related to plasticity and recovery of motor function, with little confounding effect of the massive cell death in the infarcted area.

In contrast with previous studies (Baumann et al. 2006; Gao et al. 2010), we found that small unilateral infarcts did not cause major changes in sleep and wake, although REM sleep was reduced on the first day after MCAo.

SWA in NREM sleep is linked to neuronal plasticity and improvements in motor function in the healthy brain (Huber et al. 2004; Hanlon et al. 2009). It may have a similar beneficial effect on functional recovery after ischemic injury. We found that SWA was acutely increased in all states after MCAo. However, this response was highly variable and not significant in NREM sleep and wakefulness. The increase in SWA was not related to improvement in motor function after stroke, although daily motor performance was negatively correlated to SWA in wakefulness and REM sleep

EEG spectra in NREM, REM and wakefulness were severely affected by MCAo, although the EEG patterns typical for these states remained visible. In all states, higher (>7Hz) EEG frequencies were reduced ipsilaterally after MCAo. The suppression of the faster part of the EEG spectrum by an ischemic lesion is in line with previous results in mice (Baumann et al. 2006). Similar to our results, spindle activity in the ischemic hemisphere is reduced in the acute phase after stroke in patients, and recovered in the chronic phase (Gottselig et al. 2002). Sigma band power and spindle activity in NREM are involved in learning processes in healthy subjects (Gais et al. 2002), as is fast EEG activity (Morin et al. 2008); their role in the relearning of motor skills after stroke may be similar.

We found that the amount of post-MCAo motor function improvement was negatively correlated the magnitude of the EEG change the beta frequency range in wake (17-21Hz) and REM (18-25Hz) and in NREM (7-20Hz), although daily SPR performance positively correlated to EEG power in the same ranges in NREM and REM. Thus, animals with large initial motor deficits showed the largest amount of recovery of EEG power, although their total amount of motor function recovery is less than that of animals with a smaller initial deficit. The relatively small changes in their EEG after stroke indicate a limited disruption of their motor cortical activity, allowing faster relearning of old motor skills, or finding alternate strategies. The loss of higher frequency EEG power possibly reflects a decreased ability of the motor cortex to display desynchronized activity, which reduces its ability to form the complex activation patterns necessary for the execution of fine motor skills.

The loss of power in a wide range of higher EEG frequencies in all vigilance states in the first week after MCAo may be the result of excessive tonic inhibition, which was recently shown to be involved in remodeling of peri-infarct areas and functional recovery (Clarkson et al. 2010). Excessive tonic inhibition may also account for part of the acute increase in SWA. If sufficient desynchronization of the motor cortical network is required for motor function recovery after stroke, increasing the ability of the cortex to desynchronize may improve the amount and rate of recovery. Desynchronization may be increased by increasing neuronal activity, or by reducing neuronal inhibition. Stimulants such as amphetamines (Papadopoulos et al. 2009; Lokk et al. 2010) and fluoxetine (Pariante et al. 2001; Chollet et al. 2011) have shown positive results, as have inverse agonists of extra-synaptic GABA-receptors (Clarkson et al. 2010). Timing is essential for both options, however, as premature neuronal stimulation would exacerbate excitotoxicity.

Although SWA possibly has positive effects on the re-learning of motor skills, we show a negative correlation between motor performance and SWA in wake and REM. The net depotentiation associated with SWA may lead to a further suppression of activation

patterns needed for movement execution. Moreover, as mentioned in chapter 7.1, wake SWA is associated with poor performance in conditions without brain damage.

All in all, normalization of the EEG by pharmacological means may seem beneficial for functional recovery, but with at least some state-specific effects, the implementation of such a therapy will be difficult. For example, increasing beta-range activity during NREM and REM sleep by a stimulant could have promising effects, but is problematic because NREM sleep, and to a lesser extent REM sleep, is characterized by a reduction of neuronal activity, and administration of the drug will likely wake the subject. Moreover, only ipsilateral EEG changes were related to functional recovery.

Further testing of the role of desynchronization in functional recovery would require: 1. a finer array of electrodes, to allow for direct measurements of synchrony and functional connectivity within the motor map, and 2. a vigilance-state triggered local drug delivery to affect local neuronal activity, with minimal disruption of the global vigilance state.

7.3 General conclusions

The general aim of this thesis was to gain insight into the functions of sleep by studying it in the unusual conditions of chronic sleep restriction and ischemic stroke.

The expression of sleep-like activation patterns during wakefulness in the first paper in this thesis illustrates how essential sleep is. The brain will try to compensate for the lost sleep in any way it can. Given that sleep is so essential, what is its function? The diversity of the regional responses to chronic sleep loss underscores the local nature of sleep regulation. If recovery occurs, it happens on a local level. It is possible that a daily quatum of slow waves must be met, possibly to maintain synaptic homeostasis, preferably within sleep, but if need be outside of it. Our chronic sleep restriction results do not give evidence for the function(s) of sleep, but are in line with previous results

If sleep has a function in neuronal remodeling in the healthy brain, it may play a similar role after stroke, when extensive neuronal reorganization occurs. Our results in the second article in this thesis show that a small ischemic lesion in the somatosensory cortex has mostly local effects on the motor cortical EEG and does not induce long-lasting changes in sleep-wake patterns. The EEG changes found after stroke are widespread and not limited to sleep. Correlations between sleep EEG changes and motor performance indicate that sleep may play a role in functional recovery, but the possible mechanisms remain unclear.

However, the results of both papers show the importance of regarding sleep and wakefulness as both local brain processes and global behaviors. Although the effects of a manipulation may not be directly visible in the amount of sleep or wakefulness, activity within a state may be severely altered. Moreover, sleep and wakefulness are not completely separable events. Sleep-like patterns may occur during wakefulness, just as changes in wake-like activation may be found during sleep. A strict dichotomy between these two states is too simplistic, nor can one simply be regarded as a byproduct of the other. If sleep and its functions, or wakefulness for that matter, are to be understood, they must be regarded in relation to each other on both a behavioral and neuronal level.

8. References

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9. List of abbreviations

(r)ANOVA – (repeated measures) analysis of variance

AMPA - α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, or quisqualate

AW – active wakefulness

BDNF – brain-derived neurotrophic factor

BSL – baseline

CA1, CA3 – Cornu ammonis, region of the hippocampus

Con - contralateral

D, d – day(s)

EEG - electroencephalogram

EMG - electromyogram

GABA – gamma amino-butyric acid, inhibitory neurotransmitter

GHB – gamma hydroxy-butyric acid

Ips - ipsilateral

LDTg – laterodorsal tegmental nuclei

LFP – local field potential

LTP – long term potentiation

MCAo – middle cerebral artery occlusion

NREM – non-rapid eye movement sleep

PPTg – pedunculopontine nuclei

QW – quiet wakefulness

REM - rapid eye movement sleep

SEM or s.e.m – standard error of the mean

SI – supporting information

SR – sleep restriction

SWA – slow wave activity

SWE – slow wave energy (SWA x time)

tPA – tissue plasminogen activator

10. Curriculum Vitae

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Peer- reviewed articles

Sleep homeostasis in the rat is preserved during chronic sleep restriction; *S. Leemburg, V.V. Vyazovskiy, U. Olcese, C. Bassetti, G. Tononi, C.Cirelli*; PNAS 2010; 107-36:15939-15944

Hormonal, hypothalamic and striatal responses to reduced body weight gain are attenuated in anorectic rats bearing small tumors; *L. Pourtau, S. Leemburg, P. Roux, T. Leste-Lasserre, P. Costaglioli, B. Garbay, G. Drutel, J.P. Konsman*; Brain Behav Immun 2011; in press

Sleep restriction by forced activity reduces hippocampal cell proliferation; *V. Roman, K. Van der Borgh, S.A. Leemburg, E.A. van der Zee, P. Meerlo*; Brain Research 2005; 1065(1-2):53-9.

Oral presentations

Sleep, EEG spectra, neuronal plasticity and functional recovery after stroke; *S. Leemburg, B. Gao, E. Kilic, J. Sarnthein, C.L. Bassetti*; 7th Day of Clinical Research, Centre for Clinical Research, Zurich 2008

Motor cortical plasticity and EEG spectrum after focal brain ischemia in the rat; ; *S. Leemburg, B. Gao, E. Cam, J. Sarnthein, C.L. Bassetti*; 1st Clinical Neuroscience Symposium, Zurich 2008

Sleep restriction in the rat during chronic sleep restriction; *S. Leemburg, V.V. Vyazovskiy, U. Olcese, C. Bassetti, G. Tononi, C.Cirelli*; 20th Congress of the European Sleep Research Society, Lisbon 2010

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